EXTRACTION OF PHYTOCHEMICALS FROM *Scolymus hispanicus* AND DETERMINATION OF POTENTIAL HEALTH EFFECTS

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
Izmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Biotechnology

by
BILAL AHMAD

December 2017
IZMIR

We approve the thesis of **BILAL AHMAD**

Examining Committee Members:

Assist. Prof. Dr. Ali Oğuz BÜYÜKKİLECİ
Department of Food Engineering, Izmir Institute of Technology

Prof. Dr. Erdal BEDİR
Department of Bioengineering, Izmir Institute of Technology

Prof. Dr. Oğuz BAYRAKTAR
Department of Chemical Engineering, Ege University

29 December 2017

Assist. Prof. Dr. Ali Oğuz BÜYÜKKİLECİ
Supervisor, Department of Food Engineering, Izmir Institute of Technology

Assist. Prof. Dr. Şükrü GÜLEÇ
Co-Supervisor, Department of Food Engineering, Izmir Institute of Technology

Associate. Prof. Dr. Engin ÖZÇİVİCİ
Head of the Department of Biotechnology and Bioengineering

Prof. Dr. Aysun SOFUOĞLU
Dean of the Graduate School of Engineering and Sciences

ACKNOWLEDGEMENTS

I would like to thank several people who really motivated and helped me in my MSc studies and in writing my MSc thesis. Firstly, I would like to express my sincere and special thanks to my advisor Assist. Prof. Dr. Ali Oğuz BÜYÜKKİLECİ for all of his support since the beginning of my MSc study for his motivation, sharing knowledge, friendship and endless devotion. He guided me everywhere from academic life to personal life and helped me whenever and where ever I needed his help. His help and guidance will be remembered throughout my life.

Besides my advisor, I would like to thank my co-advisor Assist. Prof. Dr. Şükrü GÜLEÇ for his great support, encouragement and help in difficult time.

I also thank Dr. Ece SÜREK, Cansu Özel TAŞCI, Nuket POLAT, Kevser SABANCI and Dr. Semanur YILDIZ for their kind help and support through out my research work. Additionally, I would like to thank my colleagues Nasar KHAN, Harpreet Singh BRAR, Massub TEHSEEN and Muhammad YASIR, for their great support, encouragement and motivation. Special thanks to my beloved friend Ayşe AZİZE for her prayers and support during difficult time. I am also too much thankful to Prof. Dr. Erdal BEDIR in the department of Bioengineering and research assistant Miss Nilgün YAKUBOĞULLARI in the department of Bioengineering for their help and guidance in TLC experiments.

Special thanks to my family members who prayed for my success, encouraged me during this research and I thank them also for believing in me and supporting me at each and every stage of life.

Me, my supervisor and co- supervisor are especially very THANK FUL to TAGEM (TAGEM /16 /AR-GE /53) for financially supporting our project. We are also thankful to Dr. Ünal KARIK for providing us plant samples throughout the the research work.

ABSTRACT

EXTRACTION OF PHYTOCHEMICALS FROM *Scolymus hispanicus* AND DETERMINATION OF POTENTIAL HEALTH EFFECTS

Golden thistle (*Scolymus hispanicus*) is an edible medicinal plant growing in Turkey. It has been in use since decades for the treatment of various disorders by local folks. In the past the extracts from the root barks of *S. hispanicus* were in use in the form of a medicine for the removal of kidney stones. Its root barks are the only eaten part and the root internal and aerial parts are considered as residues. The effect of harvesting time and plant maturity on phytochemicals composition of this plant have not been studied before. Besides that, in previous studies only the edible part was analyzed for bioactive constituents and the residues (aerial parts and root internal parts) have not been analyzed before. In this study, various phytochemicals and total antioxidant activities in the ethanol extracts of aerial parts, root barks and root internal tissues of *S. hispanicus* harvested from November 2016 to July 2017 were measured. The dominant phytochemicals were different in the roots and the aerial parts, whereas phytochemicals were influenced differently by the harvesting time. Total phenol contents and total antioxidant activities were higher in the aerial parts than the root parts, while total triterpenoid contents were higher in root barks and root internal tissues. Thin Layer Chromatography (TLC) analysis showed that there were not any free triterpenoids in the extracts, however there were glycosides, which may have contained triterpenoids. The crude extracts of *S. hispanicus* showed cytotoxic effects on Caco-2 cells growth. The results suggested that these extracts might have potential preventative effects on colon cancer.

ÖZET

Scolymus hispanicus'TAN FİTOKIMYASALLARIN EKSTRAKSİYONU VE POTANSİYEL SAĞLIK ETKİLERİNİN BELİRLENMESİ

Şevketi Bostan (*Scolymus hispanicus*) Türkiye'de gıda olarak tüketilen tıbbi bir bitkidir. Yerel halk tarafından çeşitli rahatsızlıkların giderilmesinde uzun yıllarıdır kullanılmaktadır. Geçmişte ilaç formundaki kök kabuğu özütleri böbrek taşı düşürmek için kullanılmıştır. Sadece kök kabuğu yenmekte, toprak üstü kısmı ve kök ortası atık olarak kabul edilmektedir. Toplama zamanı ve bitkinin olgunluğunun bu bitkinin fitokimyasal kompozisyonuna etkisi daha önce incelenmemiştir. Ayrıca, önceki çalışmalarda sadece yenilen kısımdaki biyoaktif bileşenler analiz edilmiş, atıklar (kök ortası ve toprak üstü kısım) daha önce analiz edilmemiştir. Bu çalışmada, Kasım 2016 – Temmuz 2017 arası hasat edilmiş *S. hispanicus'*un toprak üstü, kök ortası ve kök kabuğu etanol özütlerinde çeşitli fitokimyasallar ve toplam antioksidan aktivite ölçülmüştür. Kök ve toprak üstü kısımlarda baskın olan fitokimyasallar farklı olurken, fitokimyasallar hasat zamanından değişen seviyelerde etkilenmiştir. Toplam fenolik madde ve toplam antioksidan aktivite toprak üstü kısımda köklere göre daha yüksekken, toplam triterpenoidler kök kabuğu ve kök ortası dokularında daha yüksek olmuştur. İnce tabaka kromatografisi analizi ekstraktlarda serbest triterpenoid olmadığını göstermiştir, ancak triterpenoid içermesi muhtemel glikozitler bulunmaktadır. *S. hispanicus'un* ham ekstraktı Caco-2 hücresi büyümesinde sitotoksik etki göstermiştir. Sonuçlar, bu ekstraktların kolon kanseri üzerinde potansiyel koruyucu etkileri olabileceğini düşündürmektedir.

TABLE OF CONTENTS

APPENDICES

LIST OF FIGURES

LIST OF TABLES

Tables **Page**

CHAPTER 1

INTRODUCTION

Phytochemicals are the bioactive chemical constituents found in plants and have beneficial health effects such as protective and disease preventive properties. Usually these chemicals are produced by the plants for defense mechanism but recently, studies have demonstrated that they can also be used to protect human against various diseases. For example, triterpenoids have been reported in DNA repairing in case of colon cancer Caco-2 cell lines, Cucurbitacin B induces G2 arrest in colon cancer and ursolic acid have been reported to have antiproliferative effects in human colon and liver cancers. Similar effects have been reported by polyphenols such as, they have inhibitory effects on colorectal cancer cells, enhance growth of fibroblast cells, cause delay in cell cycle in Caco-2 cells. These biologically active compounds are produced in different parts of the plants such as roots, flowers, legumes, seeds, leaves, vegetables etc. Plants which contain these pharmacologically active compounds are termed as medicinal plants and such plants, since decades have been used traditionally for the treatment of many diseases by ancient folks in the villages. *Scolymus hispanicus* (Golden Thistle) belongs to family Asteraceae, is also one of the traditional medicinal plant found in various parts of the world and have been in use for decades for the treatment of various diseases by the local residents as first aid treatment in villages. It is biennial or perennial herb and is very spiny. It is usually grown in wild places but recently it has been cultivated in fields as well. In Turkey, it is widely spread in Aegean, Marmara, Black Sea, Mediterranean and Central Anatolia regions, and locally is called as Şevketi Bostan. In addition to medicinal uses, it is also widely used as a vegetable by the local folks due to its delicate flavour and is easily available in the local markets of Turkey specially in the areas around Izmir province, and is grown throughout the year but the best season in which it is consumed as vegetable is the spring season. According to many ethnobotanical studies *S. hispanicus* is widely known to have beneficial health effects and is used against certain diseases by local people. These beneficial health and anti-disease effects are known to be due to the presence of countless bioactive compounds such as phenolics and triterpenoids in *S. hispanicus*. It has been reported to possess strong

antioxidant, anti-carcinogenic, antispasmodic, spasmogenic, antisudorific and antibacterial activities as well. One of the very first licensed syrup from *S. hispanicus* was Lityazol Cemil discovered by Dr. Cemil Şener in 1934, and it was used for the removal of kidney stones. The production of Lityazol Cemil was continued till 1995 in Manisa Turkey, but the production was stopped due to the lack of raw material. Due to the presence of wide variety of phytochemicals in *S.hispanicus*, some reports are available, stating its anti-carcinogenic effects by interacting with various pathways in cancerous cells. As mentioned before, *S. hispanicus* is used as vegetable; not the whole plant but only its root barks are eaten and the root internal and aerial parts are considered as residues.

To our knowledge, the bioactive constituents in *S. hispanicus* cultivated and harvested in different time periods have never been analyzed before. Besides that the bioactive constituents in the aerial and root internal parts have also never been analyzed before. The aim of this study was to qualitatively and quantitatively analyze the bioactive constituents in all the three parts (aerial parts, root barks and root internal parts), to check the antioxidant activities of all the three parts of *S. hispanicus* cultivated and harvested during different time periods and then to determine the cytotoxic effects of the crude extracts of these parts by using colon cancer (Caco-2) cell lines.

CHAPTER 2

LITERATURE REVIEW

2.1 Medicinal Plants

The word medicinal plants are used for all those plants which have beneficial health effects and are used as medicine. These plants as a whole or some of their parts contain very important bioactive compounds which are used in the synthesis and development of drugs (Hassan., 2012). The bioactive compounds found inside these plants are known as phytochemicals which have beneficial health effects and are also used against the treatment of various diseases such as cancer, cardiovascular diseases, inflammatory diseases and have antibacterial and antioxidant activities as well.

Medicinal plants are in use since ancient times in order to cure many different kinds of diseases throughout the world. In developing countries rural areas, they are still used as the basic source of treatment. The primary treatment of a disease from these plants in many developing countries is still in use. (Kim., 2005 Palombo., 2006). In ancient times as there were no sufficient information about the diseases, lack of facilities and medical treatment, the people used to look for the treatment of diseases with the help of natural compounds which are present in these plants. As there were also no information available as how much fatal is the disease and what kind of specialized treatment is necessary but still people were utilizing these plants for the cure of their diseases just on the basis of their own experience (Petrovska., 2012). There are about 50,0000 plants throughout the world and amongst these plants just 1% of the plants have been investigated phytochemically (Groombridge and Jenkins., 2002). Research is also in its full swing for the discovery of new bioactive compounds but according to one report published by United Nations Environment Programme World Conservation Monitoring Centre, if the extinction rates of plants and animals continue at the same rate then after every 2 years we will lose one major drug (Palombo., 2006). As mentioned before that medicinal plants have been in use for decades but the current interest has been focused on the three main areas such as pharmacognosy, horticulture and phytochemistry. In pharmacognosy the bioactivity, mode of action and the specific

target sites are analyzed. In phytochemistry the plants are analyzed in order to check the possible bioactive compounds and then these bioactive compounds are characterized and also their structural analysis is done. In horticulture special focused is given to obtain optimum growth of the medicinal plants during the time of cultivation. Medicinal plants are also cultivated in the wild regions but there are some problems related to the harvesting of such plants in wild regions. For example, there can be loss in biodiversity, wrong identification of the plant and variations in the quality of medicinal plant may give unpleasant results (Briskin., 2000). There is also a very rare cooperation in between western medical practitioners and traditional practitioners which is due to the general thoughts that there is no scientific base for traditional medicines. Due to the need to develop new, scientific and effective drugs, pharmaceutical and scientific communities nowadays are giving special attention to traditionally used medicinal plants. They isolate, identify and purify the bioactive compounds present in these traditional medicinal plants which are then tested against different kind of diseases such as cancer, microbial infections, antiviruses, against parasitic worms etc. (Tailor et al., 2001).

2.1.1 Medicinal Plants in Turkey

Turkey is amongst the richest countries around the globe in case of plant diversity and about 10,500 plant species within the borders of Turkey have been indicated and amongst these about 30% of plant species are endemic (Guner et al., 2000 Cakilcioglu and Turkoglu., 2010). To evaluate the value of an environment of a specific area, endemism is considered to be the most important factor and as compared to other European countries the rate of endemism of plant species is higher in Turkey (Uglu et al., 2008, Tetik et al., 2013). This high rate of plant diversity is due to the geographic, climatic, topographic and edaphic factors. Many studies and surveys have also been done by different groups for ethnobotanical studies of Turkish flora. Ugulu et al., (2009) reported 108 plant species from 54 different families around Izmir province, Cakilcioglu et al., (2010) reported medicinal plants which belong to 32 families in Sivrice Turkey, Polat et al., (2011) reported 50 plants species from 25 families in Bingol district of Turkey, Korkmaz and Karakus., (2015) reported 64 plants taxa from 53 genera and 29 families in Uzumlu district Erzincan Turkey. The main aim of the

scientific community and the drug industry of the country is to utilize these plants for the production of drugs. The number of the plants which are examined for this purpose is very high but in comparison to the number of the plants which are transferred into the useful drugs are very low. Besides that, the number of licensed drugs in turkey is also very low (Tunalier et al., 2006). Amongst these plants, one of the plant known as *Scolymus hispanicus* is also in use since decades by the local folks as part of their diet and also as a traditional medicine in different parts of Turkey including Izmir province. There is small amount of experimental data available regarding the beneficial health effects of *S. hispanicus*. So the detailed study about the plant of our interest (*S. hispanicus*) is described in the following sections.

2.2 *Scolymus hispanicus*

Golden thistle (*Scolymus hispanicus*) belongs to Asteraceae family. This plant is used both as a medicine and as well as a vegetable. Mostly it is grown in non-cultivated regions but as it is one of the famous and favorite vegetable in Turkish markets there is also a need to cultivate this plant in the fields and farms (Sari et al., 2011). It is also consumed widely in Central Spain and other Spanish regions and has the credit of most loved vegetable by the people of Spain as well. It is widely used up by the people in countries such as Portugal, Morocco, Greece, Italy, Turkey and France. In short, this plant is used up by the people in all those areas where it is grown. *S. hispanicus* is a very hardy plant and it is resistant to cold. It usually prefers the soil which is rich in organic matter and can be grown best in such soil but generally it can be grown on all kinds of soils. The central part of the leaves of this plant is consumed. It is boiled and then fried with eggs, garlic, eaten in salads, soups and cured ham (Fig:2.1). Its roots are also used as a substitute for coffee and the coloring of the flowers is used alternatively to saffron (Polo et al., 2009). The young tiny stem is also used for the same purpose. Besides this, it is also used as a traditional medicinal plant in several countries including Turkey. It has been used for medical purposes in villages by ancient folks in different parts of the world including Turkey due to its medicinal properties like diuretic, lithiuretic, depurative, choleretic, against obesity and cholesterol, against cold, for the treatment of ulcer, against stomach ache, digestive etc. (Table 2.1). The antispasmodic and spasmogenic effects from the extracts of the root barks of this plant have been

reported practically by Kirimir et al., (1997) in rat ileum. It is also reported that it has antioxidant bioactive compounds which are discussed in the coming section.

Table 2.1. List of medical uses of *Scolymus hispanicus*

Area Name	Parts Used	Medicinal Use	Reference
Manisa, Turkey	Roots	Diuretic, Ulcer	Ugurlu and Secmen., 2008
Badajoz province, Spain	Leaves	Digestive	Vazquez et al., 1996
Granada province, Spain	Internal Part	Gastralgias, Cold	Benitz et al., 2010
Spain	Leaves	Gastralgias, Eye Infections	Morales et al., 2014
Izmir, Turkey	Seeds	Nephritis, Diuretic, Stomachache, Choleretic	Ugulu et al., 2009
Morocco	Roots	Hypoglycemic, Against Obesity and Cholesterol	Bnouham et al., 2002
Alaşehir Manisa, Turkey	Aerial, Leaf, Roots	Nephralgia, Kidney Stones	Sargin et al., 2013

Figure 2.1. *Scolymus hispanicus* different views. A. Blooming Plant. B. Small rosettes collected.C. Peeling labour. D. Spanish traditional dish with *S. hispanicus.* (Source: Polo et al., 2009).

2.2.1 *Scolymus hispanicus* in Turkey

Scolymus hispanicus is used widely also in Turkey. It is easily available in markets and bazars. The local names for this plant are Şevketi Bostan, though other alternative names are also used such as Altındeki, Sıradiken, Akkız, Sarıcakız, Akdiken. The height of the plant is about 70 cm and it is perennial or biennial herb, grown in most regions of Turkey such as Marmara, Aegean, Black sea, Central Anatolia, Mediteranean regions except the South-east and east part of Turkey (Tunalier et al., 2006, Davis., 1975). It is consumed as vegetable in Aegean region specially around Izmir and as well medicinaly in different parts of Turkey. Different ethnobotanical studies have been carried out to investigate the medicinal use of the plant by the local people in different parts of Turkey. The wild population of *S. hispanicus* is decreased due to over collection for edible use. However very recently, it is cultured and grown in farms due to its medical uses. The extracts from the roots barks of *S. hispanicus* has been used for the removal of kidney and bladder stones (Tunalier et al., 2006). Dr. Cemil Şener licensed the root extract of *Scolimus hispanicus* in 1934 under the name of Lityazol Cemil. Lityazol Cemil was produced in a production plant in Manisa, Turkey until 1994 and the production stopped due to lack of enough raw material (*S. hispanicus*). It was claimed that kidney stones removing effect was due to taraxasteryl acetate which is a triterpenoid (Tunalier et al., 2006).

Figure 2.2. Syrup from the root extracts of *S. hispanicus*, licensed by Dr. Cemil Şener. (Source: www.apelasyon.com/Yazi/470-lityazol-cemil-ve-sevketi-bostan).

A **B**

C **D**

Figure 2.3. Field pictures of *S. hispanicus* grown in Aegean Agriculture Research Institute, Izmir Turkey. A. Adult Plant. B. Root portion. C. Cultivation D. During harvesting time.

2.2.2 Chemical Contents of *Scolymus hispanicus*

As discussed before that *Scolymus hispanicus* has been used traditionally by the local folks for the treatment of various diseases. These medical uses of *Scolymus hispanicus* are due to the presence of certain bioactive compounds (phytochemicals) which are present inside the plant. The previous studies which have been done regarding phytochemicals in the aerial parts of *Scolymus hispanicus* reported that the aerial parts contain bioactive compounds such as flavonoids (biorobin, trifolin, saxifragin, 6,8-di-C-glucosylapigenin), Flavonoid glucosides (quercetin-3-O-galactoside, isoquercitrin, quercetin-3-O-glucoside, quercetin-5-O-glucoside, rutin or quercetin-3-O-rhamnoside). Flavonol rutinosides (kaempferol-3-O-α-L-rhamnophranosyl- β-D-glucopyranoside, Isorhamnetin-3-O-α-L rhamnopyranosyl-β-D-glucopyranoside). These phytochemicals have been reported in the flowers and leaves. From the petals of *Scolymus hispanicus*, Isorhamnetin 3-galactoside and rosmarinic acid, orientin, quercetin 5-glucoside have reported. From the chloroform and petroleum ether extracts sitosteryl- 3- β-D-glycoside, multiflorenol, multiflorenol and α amyrenon were isolated (Tunalier et al., 2006). The phenolics contents extracted from this plant were applied and the antioxidant activity was analyzed, and it was then observed that concentration of phenolic compounds of *S. hispanicus* as 10µg/ml showed about 20% antioxidant ability and DNA protection in lymphocytes, where the oxidative DNA damage was introduced by the help of hydrogen peroxide (Kapiszewska et al., 2005). Similarly, from root barks of *S. hispanicus* galactose, stigma sterol, oleanolic acid, fructose, β-sitosterol, α-amyrin acetate and α-amyrin were extracted and characterized. For the first time taraxasteryl acetate a triterpenoid and one of the most important chemical, was isolated and characterized from the root barks of *S. hispanicus* in 1997 (Tunalier et al., 2006). SARI and TUTAR., (2009) reported that the root part of *S. hispanicus* contains taraxasterol and taraxasterol acetate triterpenoids, and triterpenoids have anticarcenogenic effects and it has been reported that they stop the cell cycles in cancerous cells, the details of which are given in the next section. Similarly, as *S. hispanicus* is reported to have polyphenols (Kapiszewska et al., 2005) which have antioxidant and anticarcenogenic effects as well by causing delay in cell cycles in colon cancer cell lines. They are also reported to enhance the growth of fibroblast cells (Shirai et al., 2015).

2.3 Phytochemicals

Biologically active chemical compounds produced by plants are called as phytochemicals. They are usually the secondary metabolites produced by plants and are usually found in vegetables, legumes, fruits, nuts, grains, leaves, young stems and roots and have uncountable number of health benefits and are used against the treatment of various diseases. The extraction, characterization, purifications and then checking the beneficial health effects of such bioactive compounds *in vitro*, such as anti-inflammatory, antimicrobial, antioxidant, antiviral, diuretic, analgesic, against nephritis etc have opened new ways to discover new, cheap and easily available drugs. The extraction, characterization and purification of these bioactive compounds from plants have been summarized in the following diagram.

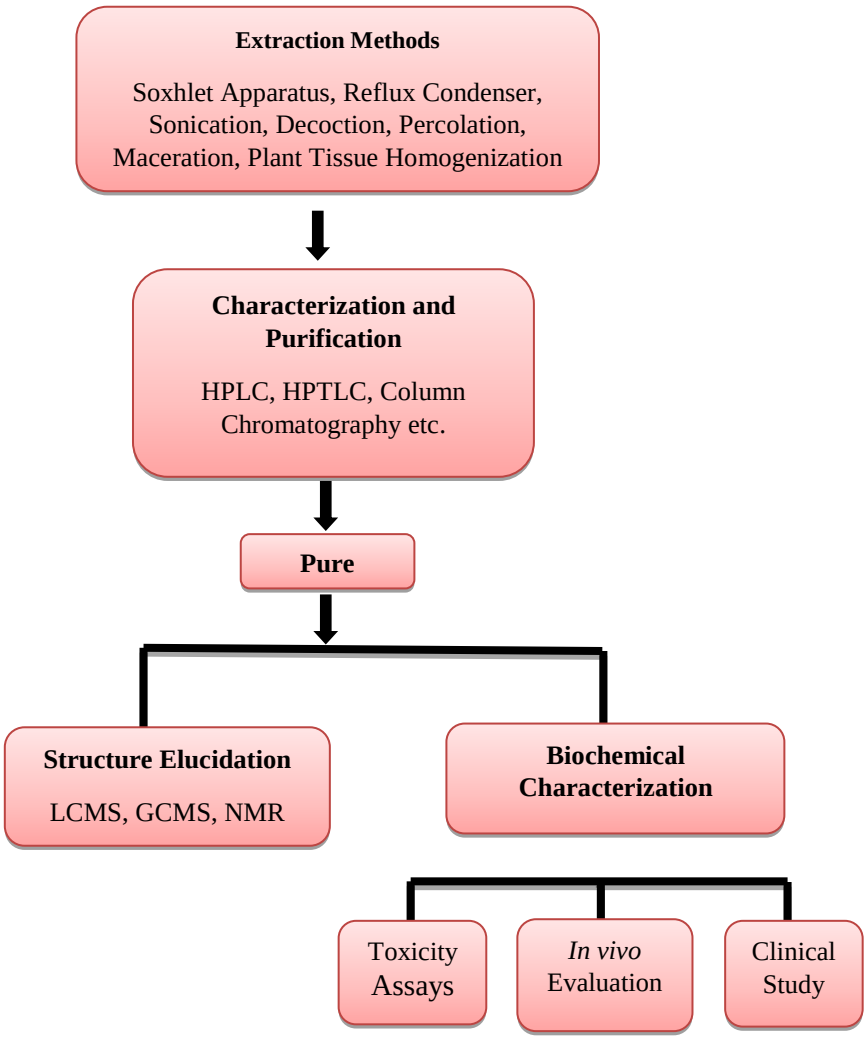


Figure 2.4. Summary of approaches of extraction, isolation and characterization of bioactive compounds from plants. (Source: Sasidharan et al., 2011).

As mentioned before that phytochemicals have countless health benefits ranging from normal daily intake to the prevention or treatment of fatal diseases. So keeping in mind their vital role in our daily life, some of the phytochemicals were tried to extract and characterize from *S. hispanicus*. So those phytochemicals have been discussed briefly in this section.

2.4 Phenolic Compounds

Phenolic compounds are the most abundant secondary metabolites found in plants naturally. Basically they are composed of one aromatic ring which is then linked to one or more hydroxyl group, that is why phenolic compounds ranges from simple phenol molecule to high molecular weight polymers (Balasundram et al., 2005). They are produced in plants by two main metabolic pathways. One is shikimic acid pathway and the other one is acetic acid pathway while when these two pathways combined then it leads to the formation of flavonoids which are naturally one of the most abundant group of phenolic compounds (Giada., 2013). They have got special attention in the recent years because they are thought to be more antioxidant as compared to any other group of phytochemicals and due to their antioxidant property they protect the body tissues under oxidative stress and reduce the risk of many fatal diseases related to oxidative stress such as cardiovascular diseases, cancer, inflammatory diseases etc. The antioxidant ability is due to the power to scavenge the free radicals and donate the electron or chelate the metal cations (Amarowicz et al., 2005). They are distributed widely in plant food such as cereals, fruits, vegetables and are considered as the most abundant antioxidants in our bodies. What makes them prominent and different than other antioxidants are the huge diversity in their molecular structures (Scalbert and Williamson., 2000). They have been reported to stop the growth and proliferation of cancerous cells by regulating those process which are responsible for the modulation of genes, such as angiogenesis and metastasis, oncogenic transformation of normal cells, growth and development of tumors. Recent studies have been focused to identify the molecular basis of cancer cells death which is induced by plant phenolics by down regulating certain kinases such as PI3K, AkT, Cyclin Dependent Kinases (CDKs), factors which regulate transcription such as NF-kB, NRF2. Moreover, the epidemiological and preclinical studies have observed *in vitro* that phenolic acids of plants are involved in the retardation of the growth of cancer cells (Anantharaju et al.,

2016). Polyphenolics are also reported to protect the viability and the ability of cells to proliferate from damages caused by UV-A radiations (Shirai et al., 2015). Moreover, polyphenol compounds of plants also give protection against UV radiations in skin fibroblast cells (Palencia et al., 2008). Phenolic compounds such as silymarin and its flavonoligans have shown chemo protective effects against toxicity induced by anthracycline in rat cardio myocytes (Chlopcikova et al., 2004). Similarly, Indap et al., (2006) reported the antiproliferative effects of the phenolic compounds (ferulic acid, curcumin, gallic acid, caffeic acid) in breast cancer MCF-7 cells. A summarized view of health benefits of plant phenolics has been given below (Figure 2.5).

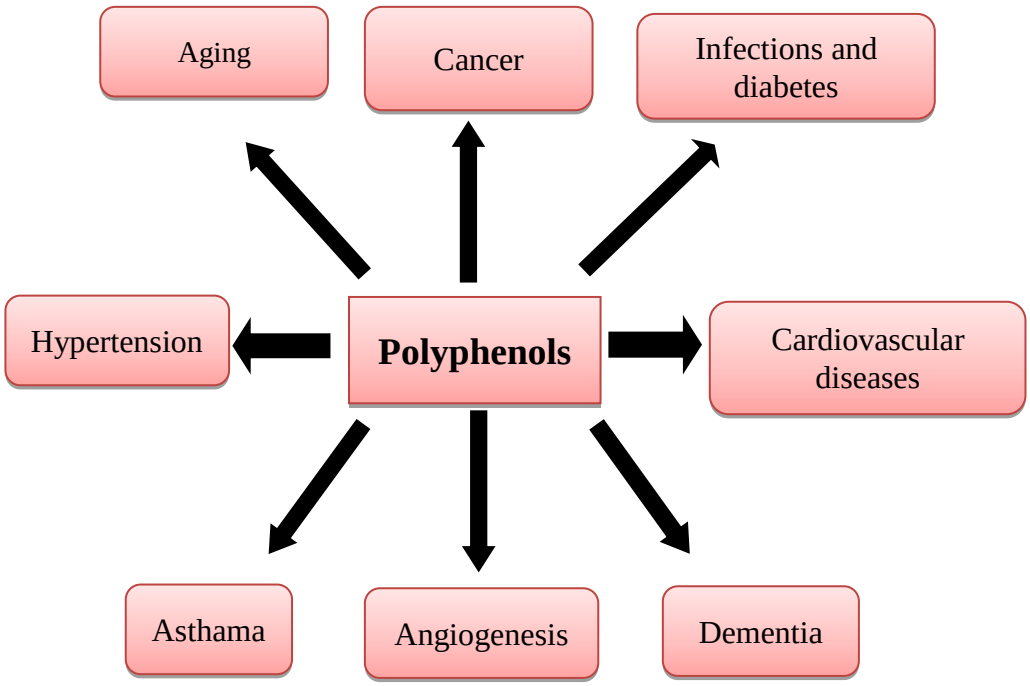


Figure 2.5. Polyphols: Health benefits and protection against certain diseases. (Source: Pandey and Riz., 2009. Moyle et al., 2015. Babu et al., 2013).

2.4.1 Classification of Phenolic Compounds

The precursors for phenolic acids are tyrosine or phenylalanine in the shikimate pathway. The key step in the biosynthesis of phenolic acids are the addition of hydroxyl groups to the phenyl ring and due to their heterogeneous structures phenolic compounds can be broadly classified into two main types which are simple phenolics and complex phenolics (Tzin and Galili., 2010).

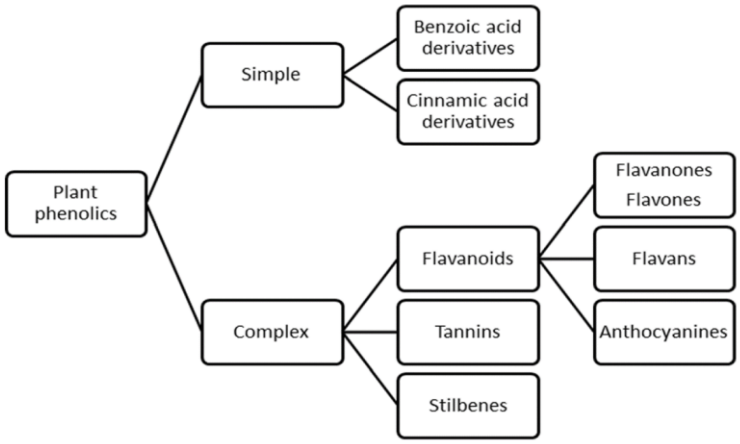


Figure 2.6. Classification of plant phenolics
(Source: Anantharaju et al., 2016)

2.4.2 Simple Phenolics

Naturally the simplest phenolic compounds with 6 and 9 carbon skeleton are benzoic acids and cinnamic acids (Giada M., 2013). These compounds contain a benzene ring to which carboxylic acid attached and also one or more hydroxyl or methoxyl group attached to benzene ring (Yang et al., 2011). For instance there are 3 Hydroxyl (-OH) group attached in gallic acid at carbon 3, 4 and 5 where as in case of syringic acid there are methoxy ($-OCH_3$) attached at 3^{rd} and 5^{th} carbon and also one hydroxyl group attached at 4^{th} carbon of benzene ring. Similarly there are two hydroxyl groups (-OH) groups attached at carbon 3^{rd} and 4^{th} in caffeic acid whereas there is one methoxy ($-OCH_3$) group attached at 3^{rd} carbon and one hydroxyl group (-OH) attached at caron 4 of benzene ring in ferulic acid (Anantharaju et al., 2016).

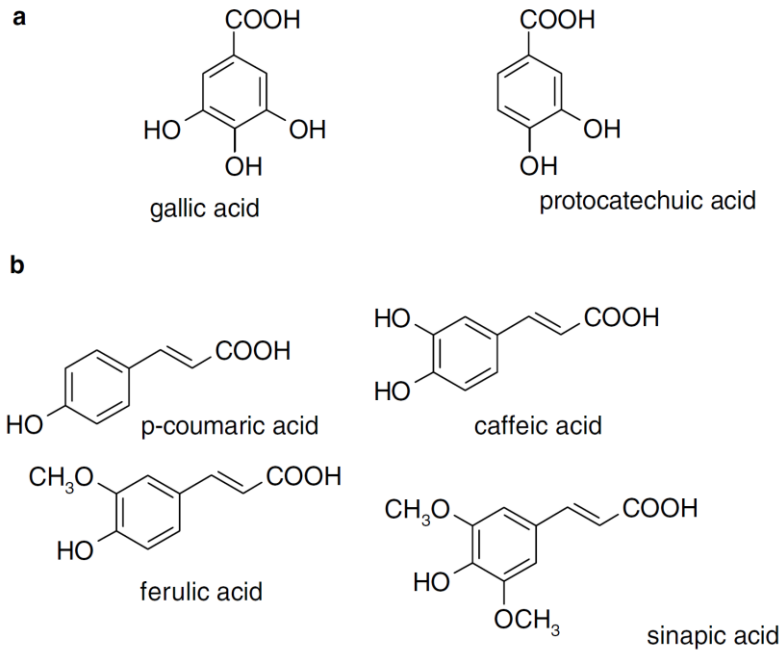


Figure 2.7. a) Examples of hydroxybenzoic acids. b) Examples of hydroxycinnamic acid. (Source: Balasundram et al., 2006).

2.4.3 Complex Phenolics

Compounds with higher molecular weights are complex phenolics. They are found mostly in the vacuoles of the plant cells. The best examples for the complex phenolics are tannins and flavonoids which are found in vegetables and fruits (Pandey and Rizvi., 2009). There are two phenolic groups in flavonoids to which oxygenated heterocyclic pyran group is attached (Kumar and Pandey., 2013). Flavonoids are further classified into anthocyanins, flavanols, flavones etc due to the oxygenated status of pyran ring. They become more complex when they are acetylated or glycosylated in addition to their primary substitution with methoxyl or hydroxyl groups (V.Cheynier., 2005).

2.5 Flavonoids

Flavonoids are the most abundant and common group of phenolics. They are complex polyphenols and their structure generally consists of 15-Carbon (C_{15}) ring in an arrangement of two benzene rings with 6-carbons each and one heterocyclic pyran ring with 3-carbons (C_6-C_3-$C_{6)}$ (Pereira et al., 2009 Kumar and Pandey., 2013). They are characterized into different classes (Fig: 2.9) i-e flavones such as epigenin and luteolin. Flavonols such as fisetin, myricetin, kaempferol, quercetin. Flavanones such as naringenin, hesperetin, flavanone. The oxidation and substitution pattern of heterocyclic pyran ring (C_3 ring) is responsible for the different classes of flavonoids. They are usually found in all plants and help in pollination by attracting insects and also play a major role in the protection of plants from Ultra-Violet radiations (Pereira et al., 2009). From medical point of view, the interest in flavonoids is increasing day by day. This is due to some of their beneficial health effects and useful properties such as antimicrobial, antiviral, antiallergic, enzyme inhibition, osteogenic, antitumor activities. Besides these properties the most important property of flavonoids is their antioxidant property which is one of the most studied property of flavonoids (Pereira et al., 2009 Cushnie and Lamb., 2005). Flavonoids also reduce the risk of stroke, cardiovascular diseases and total mortality (Hodgson., 2008). Similarly, Aherne and Brien., (2009) reported that flavonoids protect the DNA damage induced by hydrogen peroxide.

Kuo., (1996) reported the antiproliferative effect of quercetin and genistein against colon cancer. Myricetin have also been reported to have antiviral, antiplatelet, cytoprotective and hypoglycemic properties (Salvamani et al., 2013). A summary of reported health benefits of flavonoids has been given in Table 2.2.

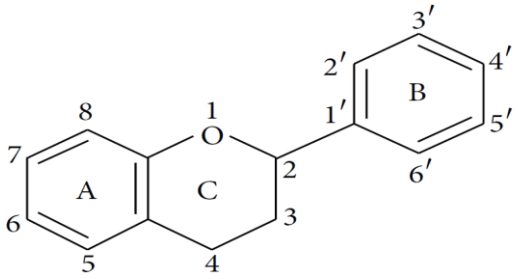


Figure 2.8. Basic structure of flavonoids
(Source: Kumar and Pandey., 2013)

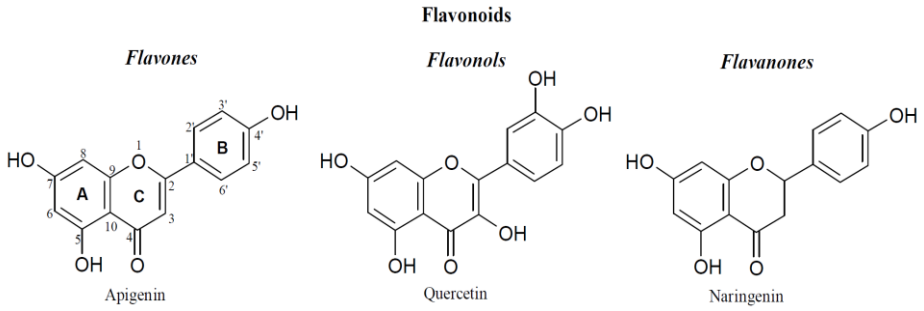


Figure 2.9. Flavonoids and its different classes
(Source: Periera et al., 2009)

Table 2.2. Summary of some of the flavonoids and their health benefits.
(Source: Salvamani et al., 2013 Kumar and Pandey., 2013)

Flavonoid	Beneficial health effects
Quercetin	Herps virus, Polio Virus, Rabies Virus, Antiobesity, Antihypertensive
Rutin	Influenza virus, Potato virus, Antiartherosclerotic
Apigenin	Herpes simplex virus type and Auzesky Virus
Naringin	Respiratory syncytial virus, Anti-inflammatory
Luteolin	Auzesky Virus
Morin	Potato Virus
Galangin	Herpes simplex virus type
Kaempferol	Antiatherosclerotic, Antihyperlipidemic, Reduce oxidative stress
Myricetın	Antiplatelet, Cytoprotective effects, Antiartherosclerotic
Fisetin	Antioxidative, Antiobesity
Gossypetin	Reduce oxidative stress, Antiatherosclerrotic, Spress LDL oxidation

2.6 Tannins

Tannins are also known as tannic acids and are polyphenols which are usually soluble in water. They are found in plants and have been in use as feed and food. They are found in legumes, millets, sorghum, dry beans, winged beans etc. Besides these, fruits are also rich source of tannins. Fruits such as grapes, cranberries, grapes, dates, plums, bananas and strawberries contain a good amount of tannins. Tannins present in vegetables are water soluble polyphenols and their molecular weight ranges from 500 to 3000 D. Presence of large number of hydroxyl and other functional groups usually 1 or 2 per 100 D are responsible for the cross linkage of tannins with other macromolecules and proteins. Tannins are classified into two main groups. One is hydrolysable tannins and the 2nd one is non hydrolysable or condensed tannins (Chung et al., 2010). The derivatives of gallic acids are hydrolysable tannins. The esterification of gallic acid to agaloyl and the core alloy group are then further oxidatively linked or maybe esterified to make more complex form of hydrolysable tannins. One of the most common example of simplest hydrolysable tannins are Gallo tannins which are polygalloyl esters of glucose. Condensed tannins are basically polymeric flavonoids. They are oligomeric and polymeric proanthocyanidins which contain catechin units. Biosynthetically, the formation of condensed tannins involves the successive condensation of the single building blocks when degree of polymerization between two and greater than fifty being reached (Khanbabaee and Ree., 2001). The general structure of hydrolysable and condensed tannins has been given in (Fig: 2.10). Tannins have also uncountable health benefits. Many reports showed that there is negative relation in between tannins consumed in tea and occurrence of cancer. They have also been reported to decrease the activities and number of many mutagens. The antimicrobial activities of tannins have also been well reported, in which it has been shown that the growth of many microorganisms such as bacteria, yeast and viruses have been inhibited by tannins which proves their antimicrobial activities. Chung et al., (2010) reported the inhibitory effects of tannins on aquatic bacteria, food borne bacteria and off flavor producing microorganisms.

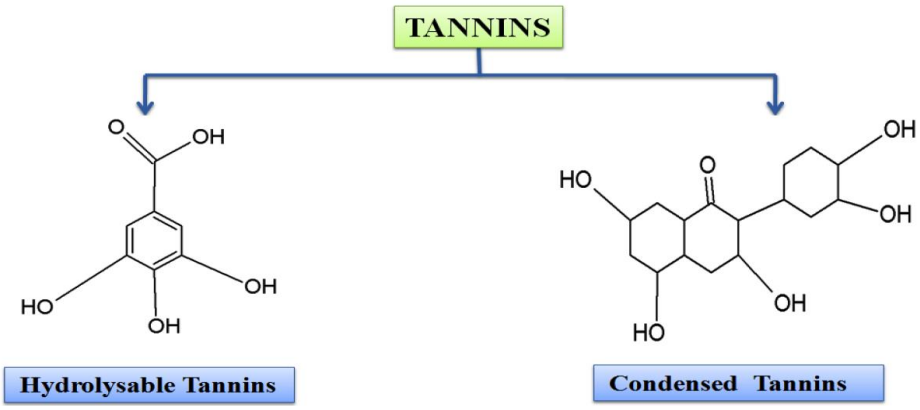


Figure 2.10. Tannins and its different classes
(Source: Ghosh et al., 2015)

2.7 Triterpenoids

Triterpenoids are amongst the most important and largest group of secondary metabolites found in plants and are also known as multifunctional secondary metabolites due to their diverse structures and properties. They are formed by the cyclization of a linear molecule squalene which is a triterpene hydrocarbon and a starting metabolite for all the steroids. They are basically tetracyclic or pentacyclic and are recognized mainly by the diversity in their structures. There are three main classes of triterpenoids which are oleane, ursane and lupane (Fig:11). Oleane family consists oleanolic acids, β-amyrin and erythrodiol. The ursane family consists of uvaol and ursolic acid. Lupane family contains botulin, lupeol and betulinic acid. These divisions among different triterpenoids depend on their parent skeleton. The main triterpenoids acids found in plants are ursolic acids and oleanolic acids (Lesellier et al., 2012). They are found in different plants and as well as in wax like coatings of herbs and fruits. They are found both in free forms and also conjugates of esters and glycosides called sapponins. As they are widely present in medicinal and edible plants, that is why they are considered as one of the most important part of human diet (Szakiel et al., 2012). According to one estimate there are more than 20,000 triterpenoids exist in nature (Bishayee et al., 2011). Due to their low toxicity level and wide range of pharmacological activities triterpenoids have got special attention and various studies have been carried out and some are in progress to identify their role in the prevention of certain diseases (Wei et al., 2015). Pitchakarn et al., (2011) reported the inhibition of growth and induction of apoptosis in prostate cancer by a triterpenoid kuguacin L.

Fukumitsu et al., (2015) reported the anti-inflammatory and antiarthritis effects of maslinic acid which is a triterpenoid in RAW 264.7 cells. Similarly, Ramos et al., (2010) reported the protective effects of ursolic acid and luteolin against H_2O_2 induced DNA damage and also the enhancement of DNA repair in colon cancer (Caco-2) cells. Recently Yang et al., (2015) reported the anti-atherosclerotic and cardiovascular effects of taraxsterol against H_2O_2 induced cell injury in human umbilical vein endothelial cell injury. Summarized form of several triterpenoid compounds and their reported health effects have been given in table 2.3.

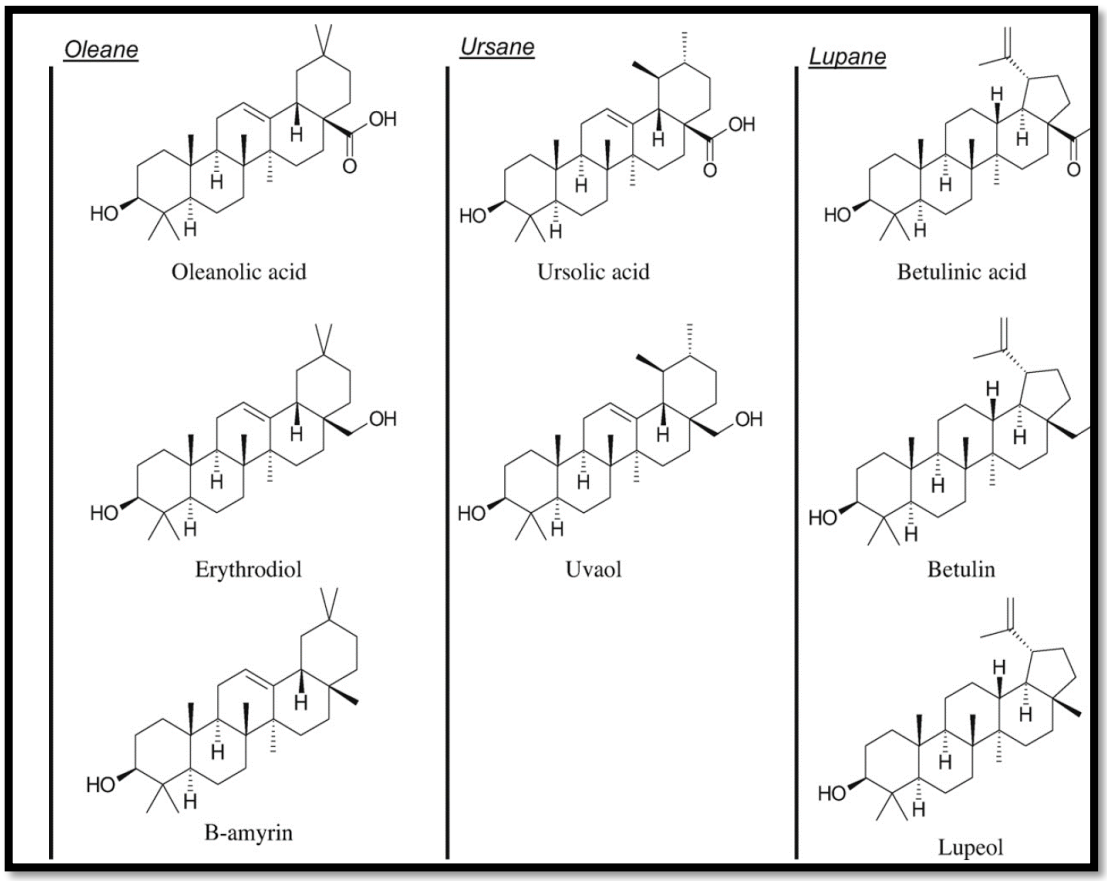


Figure 2.11. Structure of 8 pentacyclic triterpenoids
(Source: Lesellier et al., 2012)

Table 2.3. A summary of health benefits of triterpenes.
(Source: Han and Bakovic., 2015)

Oleane	Ursane	Lupane
Antioxidant	Reduced levels of urinary F2-isoprostanes	Antioxidant
Antiproliferative	Reduced levels of plasma TNF- α	Cardiotonic
Attenuation of myocardial apoptosis	Decrease in superoxide production	Antihyperlipidemic (antiatherosclerotic)
Mediation of anti-apoptotic and	Reduction of LDL and apolipoprotein B-100	Anti-inflammatory
Antioxidative activities against myocardial damage	Induction of HDL and apolipoprotein A-I concentrations	Alteration of the tissue redox system
	Reduced serum cholesterol levels and atheroma development	Decrease of ROS levels
Inactivation of the CHOP-induced Puma upregulation	Reduced risk of CVD	Induction of CAT and SOD activities
	Antioxidant	Mediation of NO synthase through
Reduction of the Tumor necrosis factoralpha	Anti-inflammatory	targeting (PI3K)/Akt and TPA
(TNF-α) promoter activity	Protection against biotic stress	Reduction of total cholesterol,
Reduced risk of atherosclerosis	Inhibition of COX-2 enzyme by 10%	triglycerides, and phospholipid levels
Reduced risk of CVD	Inhibition of Th1 responses	
Beneficial effects on oxidative stress andinflammation	Suppression of myocarditis and conferring protective effects on crdiomyopathies	
	cardiomyopathies	
Prevention of myocardial ischemia	Increase in ANP levels	
Reduced blood cholesterol levels	Increased regulation of cardiovascular homeostasis	
Regulation of Mcl-1 pro-apoptotic protein	Reduction of oxidative stress, HBP flux, and apoptosis in heart cells	
and restoration of intracellular redox state	Release of NO	
Reduced blood cholesterol levels	Inhibition of excessive glycogenesis	
	Induction of cellular glycogen context	
	• Normalization of caspase expression/activation	
	• Increase of Bcl-2/Bax ratio to modulate apoptosis	
	• Attenuation of LPS-induced translocation of NF-κB p65 subunit to the	

2.8 Colon Cancer

Cancer is a disease which does not obey the normal rules and regulation of normal cell division and as a result of which abnormal cell division occurs. The second cause of death after cardiovascular diseases is cancer in western countries. Round about 10 million people around the globe are diagnosed with cancer every year and 6.2 million are dying due to cancer worldwide (Araujo et al., 2011 Khuhaprema and Srivatanakul., 2008). Throughout the world the second cause of morbidity and mortality is the colon cancer. Colon cancer is one of the type of cancer which begins in colon or rectum. Many of the colon cancers are adenocarcinomas which form a polyp in the inner wall of colon or rectum. Two major symptoms of this diseases are blood in the stool and change in bowl habits. There is no single cause of colon cancer and there are many factors which are involved in its cause but age is one of the main risk factor, as the chances of colon cancer increases with the age. It has been shown in epidemiological studies that there is inverse relationship between colon cancer and diet rich in natural bioactive compounds such as fruits, vegetables, fibers etc (Araujo et al., 2011). Amongst all the cancer types worldwide, colon cancer is the 3rd common type of cancer and fourth most common cause of death. Men and woman are equally effected by this disease but the incidents in woman are a bit lower than man (Pampaloni et al., 2014). Canada, United States, New Zealand, Australia and some parts of Europe have highest incident rates of colon cancer while countries with low incident rates include China, India, South America and parts of Africa (Haggar and Boushey., 2009). Few years back there were intense changes in the treatment of colon cancer but the treatment by chemicals (drugs) and radiation have proved toxic and adverse side effects and were far from satisfaction therefore treatment by natural anticancer agents have already begun, especially medicinal plants which are the reservoirs of anticancer molecules (Bandopadhyaya et al., 2015). The treatment by help of naturally occurring anticarcinogenic chemicals have got special attention due to their inhibitory effects on colon cancer or any other type of cancer with lowest toxicity. Now a days round about 60 % of the drugs used for treating cancer are directly or indirectly comes from natural resources i-e medicinal plants. These antitumor drugs cause apoptosis which is pre planned cell death and in such cases the contents inside the cells do not cause any pressure on cell membrane and hence no lysis occurs as a result of which the nearby

cells will not be harmed and there will be no inflammation as well. The two most important morphological changes in apoptosis are the condensation of chromatins and the fragmentation of nuclear material specially DNA. As recent studies have shown that herbal medicine induces apoptosis in cancerous cells which is one of the most ideal result for naturally occurring antitumor drugs and that is why special attention have been made to search for apoptosis causing naturally occurring antitumor drugs (Behzad et al., 2016). As selection of a medicinal plant or some of its part for the purpose of drug discovery is totally dependent on the ethnobotanical study of that plant so that is why after studying the uses of *S. hispanicus* as traditional folk medicine in Turkey and also in some other countries (Table 2.1), it was then decided to apply the crude extracts of *S. hispanicus* on colon cancer (Caco-2) cells and check the cytotoxicity and potential preventative effects of the crude extracts of *S. hispanicus*.

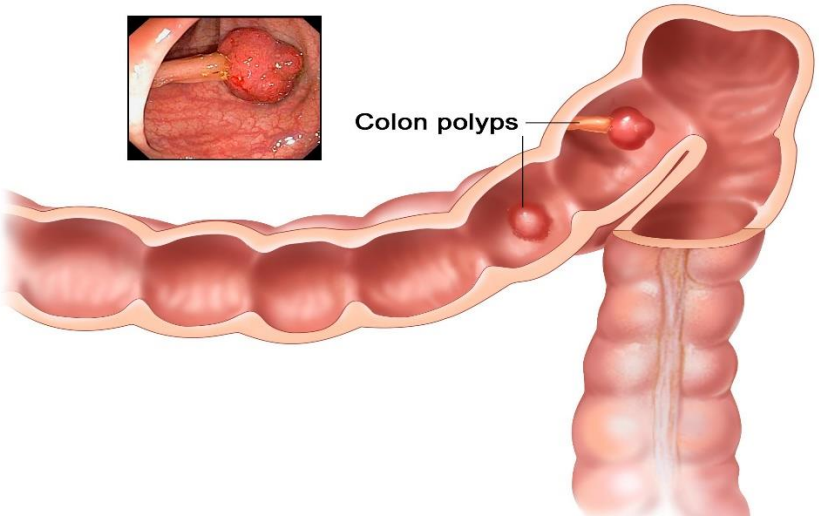


Figure 2.12. Polyps in the colon.
(Source: www.nih.gov)

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

3.1.1 Chemicals

All the chemicals which were used in this study were in their analytical standard form and no other methods such as purification were performed. The chemicals used in different experiments are given in Table A.1 of Appendix A. Deionized water was used in all experiments where needed.

3.1.2 Plant Samples

All the plant (*Scolymus hispanicus*) samples were provided by the Aegean Agriculture Research Institute Republic of Turkey, Ministry of Food, Agriculture and Live stock during different harvesting periods from November 2016 to July 2017 throughout the year. All the plant samples were provided in fine grinded dry forms. (Drying was carried out in a drying chamber at 60 °C for 48 h). The samples were from three different parts of the plant which were, root barks, root internal and aerial parts of the plant including leaves and upper stems. After receiving the samples, they were stored at room temperature in glass bottles till further use.

3.2 Methodology

3.2.1 Extraction of Phytochemicals

The extraction of phytochemicals from the dried plant samples was performed using 90% aqueous ethanol, as the extracting solvent (Figure 3.1). The extraction ratio of the extracting sample and extracting solvent was 1:10. For November samples 15 g of plant samples were extracted by using 150 ml 90% aqueous ethanol, and for the rest

of the plant samples 25 g of plant samples were extracted by using 250 ml 90% aqueous ethanol. Round bottom extraction flask was placed in a water bath on the heater-stirrer. The temperature in the bath was measured continuously by the temperature probe and maintained at 40 ℃ and regularly checked after 30 min. In the condenser, tap water was circulated to keep the the condenser cool. The extraction lasted for 4 hours. The second method for extraction of phytochemicals from *S. hispanicus* was infusion. The dried and ground *S. hispanicus* (whole plant) was provided in infusion bags containing 2.5 g of plant. The bag was kept in 100 ml of boiling water for 15 min and was dipped in and out of the water 5 times after every 5 min. The filtrate obtained was then stored at -20 ℃ till further use. All the methods which were used for the quantitative analysis and antioxidant activities were same both for the dried plants sample extracts and infusion extracts of *S. hispanicus.*

Figure 3.1. Extraction set up

3.2.2 Filtration

After extraction, all the samples were filtered through a fine filter paper (Whatman of pore size 11µm). Briefly, the filter paper was placed in a porus funnel which was fixed on a 500 ml flask connected to a vaccum pump. The vaccum pump was used to increase the speed of filtration by creating vacuum. All the liquid extracts along with the extracting solvent were collected in a 500 ml flask and the waste residues of the plant samples were dried in an oven at 100 °C and weighed laterly by standard weigh scale.

3.2.3 Evaporation of the solvent

The solvent was evaporated in a rotary vacuum (Heidolph, Germany). The extract along with the solvent was kept in a round bottom flask and fixed it into the evaporator. The temperature was kept at 40 ℃ and the pressure of the vacuum pump was set in between 100 – 120 Pascals. About 90-95% of the extracting solvent (90% aqueous ethanol) was evaporated and collected in another round bottom flask connected to the condenser tube in the evaporator leaving behind only the liquid extract of the plant sample.

3.2.4 Lyophilization

To get the extracts in solid powdered forms, all the liquid extracts were further lyophilized in a Lyophilizer (Labconco-freezone 18, USA). Before lyophilization the samples were diluted with deionized water in 50 ml falcon tubes to overcome the amount of left over ethanol in the liquid extracts, and all the samples were then kept overnight in a freezer (ULT1786-5-V39, USA) at -80 ℃. After complete freezing, the samples were then taken out and kept in a lyophilizer for almost 48 h at a pressure of 0.014 mbar and -54 ℃. After 48 h, all the samples were completely dry and weighed by using standard weigh scale. The dried samples were kept in a refrigerator at - 4℃ till further use.

3.3 Qualitative Analysis of Phytochemicals

Qualitative analysis of different phytochemicals from aerial parts, root barks and root internal parts of *Scolymus hispanicus* were carried out by using standard protocols from the literature. The extracts after lyophilization were dissolved in distilled water to make the stock solutions of concentration 10 mg/ml.

For the qualitative screening of flavonoids, 1 ml of the reconstituted lyophilized samples (concentration 10 mg/ml) was mixed with 1.3 ml of 1% potassium hydroxide and the color change was observed (Khan et al., 2011). Appearance of dark yellow color indicated the presence of flavonoids.

The detection of triterpenoids in the ethanolic-water extracts of *S. hispanicu*s was done by using the method of Gupta et al., (2013). To the reconstituted lyophilized extracts few drops of concentrated sulphuric acid were added and the change in color was observed. The appearance of yellow color precipitate in the bottom indicated the presence of triterpenoids.

Alkaloids were detected by following the procedures mentioned by Iqbal et al., (2015). 2 g of potassium iodide and 1.27 g of Iodine were mixed with deionized water and then by addition of further water the volume was adjusted to 100 ml. Then few drops from this mixture were added to the reconstituted lyophilized extracts by the help of pasteur pipette. A brown colored precipitate indicated the presence of alkaloids.

For protein detection Xanthoproteic Test was carried out. Few drops of concentrated nitric acid were added to the reconstituted lyophilized extracts and appearance of yellow color indicated the presence of proteins (Tiwari., 2011).

3.4 Quantitative Analysis of Phytochemicals

Quantitative analysis of the bioactive compounds in *S. hispanicus* was carried out by following standard procedures.

3.4.1 Total Phenol Contents

Total phenol contents from the aerial parts extracts of *S. hispanicus* were determined by Folin-Ciocalteu reagents as described by Surek and Erdil., (2014) Assanga et al., (2015). Small scale modifications were made while determining the total phenol contents of the plant extracts. Briefly, from stock solutions (10mg/ml concentration) of plant extracts, 100 and 50 µl of stock solutions were taken in test tubes in triplicates. They were then diluted 5 and 10 times and the volume was adjusted to 500 µl. Then 2.5 ml Folin reagent (prepared by diluting the commercial solution 10 times with distilled water) was added. After three min 7.5% (W/V) sodium carbonate solution was added and the color of the extracts turned darkish blue compared to blank which was an indication for the presence of phenols. The same color was also obtained while doing the experiment with standard phenolic acid (gallic acid). The mixture was mixed with the help of vortex (ISO Lab, 622.01.001, Turkey). After keeping the mixture in dark for 1.5 h the absorbance was read using the spectrophotometer (Thermo scientific, USA) at 765 nm. Gallic acid (0.02 - 0.1 mg/ml) was used as standard for the preparation of a calibration curve and all the values were expressed as mg gallic acid equivalent (GAE) per 100 mg of lyophilized extracts.

3.4.2 Total Flavonoid Contents

Total flavonoid contents were determined by following the methods of Viuda-Martos et al., (2011) and Sultana et al., (2012). 500 µl of aerial parts extracts with concentration 10 mg/ml was taken in a test tubes and 0.150 ml $NaNO_2$ (5%) solution was added and mixed well by vortex. After waiting for 5 min 0.150 ml $AlCl_3$ (10%) solution was added, after one minute 1ml of 1M NaOH solution was added and in the end 1.2 ml deionized water was added. The whole solution was mixed well by vortexing thoroughly and finally the absorbance was read at 510 nm. Methanol was used as blank

and quercetin with different concentration (0.2 – 1 mg/ml) was used as standard for making the calibration curve. All the values calculated, were expressed as mg quercetin equivalent (QE) per 100 mg of lyophillized extracts.

3.4.3 Total Tannin Contents

Total tannin contents in the aerial parts extracts of *S. hispanicus* were determined by the methods adopted by Kar et al., (2011). Briefly 0.5 ml of the extracts was taken in test tubes and then 1ml freshly prepared in ice bath vanillin- sulphuric acid (1% vanillin in 7 M $H_2SO_{4)}$ was added to the extracts. The mixture was mixed properly by vortexing and then the absorbance was read at 500 nm against blank. Deionized water was used as blank. Catechin with five different concentrations ranging from 0.02 - 0.1 mg/ml was used as standard for the preparation of calibration curve and all the values were expressed as mg catechin equivalent (CE) per 100 mg of lyophilized extracts.

3.4.4 Total Triterpenoid Contents

For the quantitative analysis of total triterpenoid contents of *S. hispanicus* the method used by Wie et al., (2015) was followed. 100 µl of pure plant extracts from root barks and root internal parts were taken in test tubes and 500 µl freshly prepared solution of 5% (W/V) vanillin- acetic acid was added to it and vortexed. After this, 900 µl of pure concentrated sulphuric acid was added and vortexed again. After vortexing the solution was incubated at 70 ℃ for 30 min in a water bath (Termal, J11880KD, Turkey). After 30 min the solution was taken out and allowed to cool at room temperature for 20 min. Finally, the solution was diluted to 5 ml by adding concentrated acetic acid, mixed and the absorbance was read at 573 nm. Ursolic acid at different concentrations ranging from 0.2 - 1.0 mg/ml was used as standard for the preparation of calibration curve and all the results were expressed as mg ursolic acid (UAE) equivalent per 100 mg of lyophilized extracts.

3.4.5 Total Antioxidant Activity

For the determination of total antioxidant activity from the aerial parts, root barks and root internal parts of *S. hispanicus*, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay was used (Miller and Evans., 1997 Huang et al., 2005). Briefly ABTS reagent was prepared by dissolving 100 mg of ABTS in 100 ml of deionized water. Potassium persulphate solution ($K_2S_2O_8$) was prepared by mixing 19 mg of potassium persulfate ($K_2S_2O_8$) in 1 ml of deionized water. Then these two solutions (ABTS and potassium persulphate) were completely mixed and kept in complete dark for at least 17 h to form radical. 0.05 M KPi buffer solution was prepared by mixing 0.05 M dipotassium hydrogen phosphate (K_2HPO_4) and 0.05 M potassium dihydrogen phosphate (KH_2PO_4). The PH of KPi buffer was kept constant at 8.0. After preparing all the reagents and buffers, the ABTS reagent solution and KPi buffer were mixed until dark blue color appeared. First the absorbance of this mixture was measured. The absorbance of this mixture should be in the range of 0.9 - 1.0. After this 100 µl of the sample was taken in a test tube and then 1 ml of ABTS reagent and KPi buffer mixture solution was added and vortexed for 15 seconds. Then after waiting for 45 seconds the absorbance was measured at 734 nm against water. Standard curve was prepared by using trolox (0.02 - 0.1 mg/ml concentrations) as standard and all the values were expressed as trolox equivalent (TEAC) per 100 mg of lyophilized extracts.

3.5 Thin Layer Chromatography (TLC) Analysis

Thin Layer Chromatography (TLC) analysis was done to analyze some of the bioactive compounds present in *S. hispanicus* by using TLC plates (Silica gel 60 F_{254}, Germany). Two different solvent systems were used for the analysis of different phytochemicals groups. Firstly, a TLC solvent system was developed by using hexane and ethyl acetate (8:2) to identify the non-polar compounds present in *S. hispanicus*. Standard solutions of taraxasterol, taraxasterol acetate and ursolic acid were prepared by dissolving them in chloroform individually. After the saturation of the TLC solvent tank with the solvent system, the standards were spotted on the TLC plate and then the TLC plate was dipped into the TLC solvent tank. The solvent sysytem started moving upward in the TLC plate and after waiting for few minutes the plate was taken out of the

solvent tank, dried with a hair dryer and then visuallized under ultra violet (UV) light. After that, lyophillized extracts of all the three parts of *S. hispanicus* were also dissolved completly in chloroform and spotted on the TLC plate along with the standards. The same solvent system (hexane and ethyl acetate) was used for the analysis of the lyophillized extracts of *S. hispanicus*.

The second TLC solvent system was developed which was comparatively more polar than the previous solvent system. Chloroform, methanol and water in the ratio of 61:32:7 were used to develop the second solvent system. All the three lyophillized extracts of aerial parts, root barks and root internal parts of *S. hispanicus* dissolved in chloroform and then spotted on TLC plate. The TLC plate was then dipped into the solvent system. After waiting for few minutes, the TLC plate was taken out of the tank and dried with the help of a hair dryer. It was then visuallized under UV light. The TLC plate was then sprayed by sulphuric acid and then then dried again.The purpose of spraying with sulphuric acid was to make the bands more visible.

3.6 Culturing the Caco-2 Cells

The most suitable and acceptable route for the administration of drugs to the body is the gastrointestial tract. The reason is that its absorbtion rate is higher as compared to other routes of administrations (Meunier et al., 1995). For smaller molecular weight drugs the most preffered route of administration is the oral administration. There are many factors which effects as a whole, the bioavailibility of the orally administrated drugs such as morphological and physiochemical properties of drugs and the biochemical state of intestinal epithelial as well. The culture model of Caco-2 cell lines was firstly introduced in 1990 and now it is widely used for the determination of the characteristics of the intestinal transport of drugs and also to predict the limited absorption of drugs. These cell lines form confluent monolayers under standard culturing conditions upon spontaneous differentiations (Ingels and Augustijns., 2003). Colon cancer Caco-2 cell lines have numerous properties and in order to study the intestinal epithelial differentiations, these cells have been widely used in such studies since long time because they have the ability to express biochemical and morphological features of adult differentiated enterocytes (Caro et al., 1995). One important feature of this cell line is that when they are grown on nitrocellulose filters or

plastic dishes then their morphologic characteristics are same just like the normal enterocytes (Ismael et al., 1989). Differentiated human intestinal Caco-2 cell lines are also used for the toxicological and pharmacological studies as *in vitro* models for the studies of intestinal barrier functions (Zucco et al., 2005). Similarly, Chantret et al., (1988) reported that when different types of colon cancer cell lines were grown and compared then amongst all those cell lines, only Caco-2 cell lines showed the most suitable and better structural and morphological characteristics for the study and investigation of nutrient metabolism.

In our study, Caco-2 cell line (ATCC Number: HTB-37) were purchased from ATCC cell culture collection. Minimum Essential Medium (MEM) with Earles salts, L-glutamine and sodium bicarbonate was used for the growth of Caco-2 cell line. 10^6 cells in 10 ml of growth medium were maintained in 100 mm diameter petri dish and humidified atmosphere at 37 °C with 5% CO_2 in the cell culture incubator (Thermo Fisher Scientific, 3404, USA) and regularly observed. After reaching to 90% confluency, the cells were splitted to new plates in the ration of 1:5. The stock of Caco-2 was prepared with freezing solution (5% DMSO and 95% DMEM medium, contains 20% FBS) stored in liquid nitrogen and cells were taken when they were needed. The passage number of Caco-2 cells was always kept between 20-30.

3.7 Preparation of Plant Extracts

Initially 5 mg/ml of plant stock solutions were prepared in deionized water from aerial parts, root barks and root internal parts of November and May samples. The crude extracts of November and May samples were chosen to apply on Caco-2 cell lines. The reason for choosing November and May samples was that they gave high amount of phenolic compounds and triterpenoids as compared to other months samples of *S. hispanicus*. From the stock solutions (5 mg/ml), different working solutions of different concentrations (25, 50, 100, 200, 400 µg/ml) were prepared with MEM culture media in eppendorf tubes. The purpose of using different concentrations was to determine the minimal and maximal effective doses of the crude extracts of *S. hispanicus.*

3.8 Treating the Cells with Plant Crude Extracts

After 24 h the already cultured cells in 96 wells plates were examined under microscope (VWR, UK) and they were 80% confluent. The media was then removed from 96 wells plate leaving behind only CaCo-2 cells. Then 200 µl of already prepared different concentrations of the plant crude extracts were added to each well in triplicate. In 4 wells of 96 wells plate, only 200 µl of culture media was added and was considered as negative control. The plate was again incubated for 48 hours at 37 ℃ under 5 % CO_2 in humidified conditions. After 24 h the cells were examined and some morphologic changes were seen, so it was decided to incubate them further for 12 h.

CHAPTER 4

RESULTS & DISCUSSION

4.1 Results

4.1.1 Extraction

The purpose of the extraction was to get only the pure bioactive compounds and to get rid of waste and non-useable residue. So as a result of extraction different liquid extracts were obtained leaving behind the waste residue. The color of the extracts from the aerial parts were green and the extracts from ground parts (root barks and root internal parts) were yellowish in color.

4.1.2 Evaporation

The presence of alcohol does not allow the extracts to be lyophilized, that is why all the extracts after obtaining in liquid form were subject to evaporation to minimize the amount of ethanol in the extracts. As a result of evaporation, the liquid extracts became more concentrated with only a small amount of ethanol and water and were ready to lyophilize.

4.1.3 Lyophilization

All the samples which were stored in a freezer at -80 °C were lyophilized. In the beginning of lyophilization the samples started to coming out of the falcon tubes which was the indication that ethanol is still present in the samples. To overcome this problem all the samples were diluted about 5 times with deionized water to overcome the effect of ethanol. After dilution the samples were lyophilized and interestingly, different extract yields were obtained from the different samples of the same plant. The extract yields after lyophilization are given in Table 4.1.

Table 4.1. Different extract yields of *S. hispanicus* from different samples

Plant Sample	**Extract Yields (%)**		
	Aerial Parts	Root Barks	Root Internal
November	9.73	8.73	14.26
January	3.28	4	10.8
March	4.44	4.88	6.88
May	7.48	13.13	14.26
July	3.32	6.8	12

4.1.4 Qualitative Analysis of Phytochemicals

Phytochemicals are the bioactive compounds present inside the plants and most of the phytochemicals are secondary metabolites produced by plants for defense mechanism. Some of them may have possibly health benefits and various anti-disease effects as well. Therefore, the phytochemicals present in *S. hispanicus* were screened using qualitative assays.

So in order to check the availability and non-availability of these important bioactive compounds in *S. hispanicus*, different qualitative screenings were done by using standard procedures from the literature. After the confirmation of the presence of these phytochemicals in different parts of *S. hispanicus*, we proceed further and analyzed these compounds quantitatively. All the results of qualitative screening of phytochemicals in *S. hispanicus*, are given in Table 4.2 and Figure 4.1 - 4.4

Table 4.2. Qualitative analysis of phytochemicals

Bioactive compounds	***Scolymus hispanicus* Parts**		
	Aerial Parts	Root Barks	Root Internal Parts
Phenols	+	+	+
Flavonoid	+	+	+
Triterpenoids	+	+	+
Proteins	+	+	+
Alkaloids	+	+	+

+: Present -: Absent

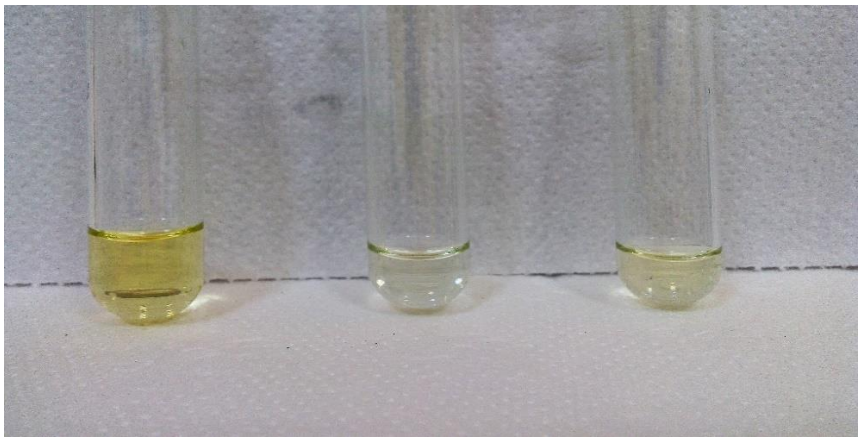


Figure 4.1. Proteins detection in different parts of *Scolymus hispanicus.* From left to right: Aerial parts, root barks and root internal parts.

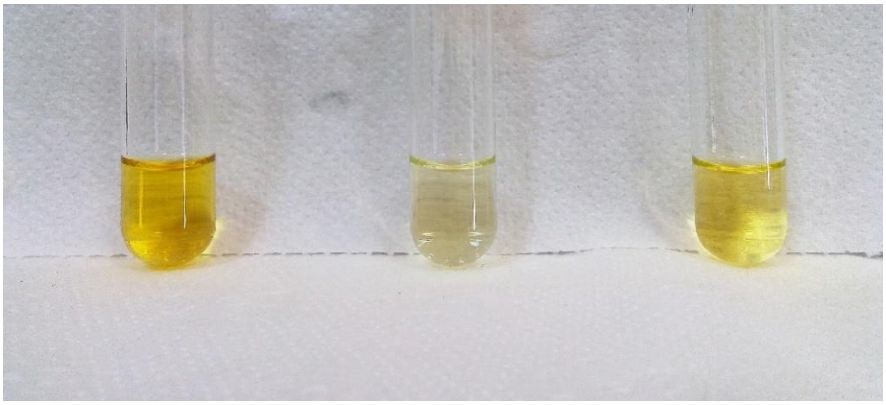


Figure 4.2. Flavonoids detection in different parts of *Scolymus hispanicus*. From left to right: Aerial parts, root barks and root internal parts.

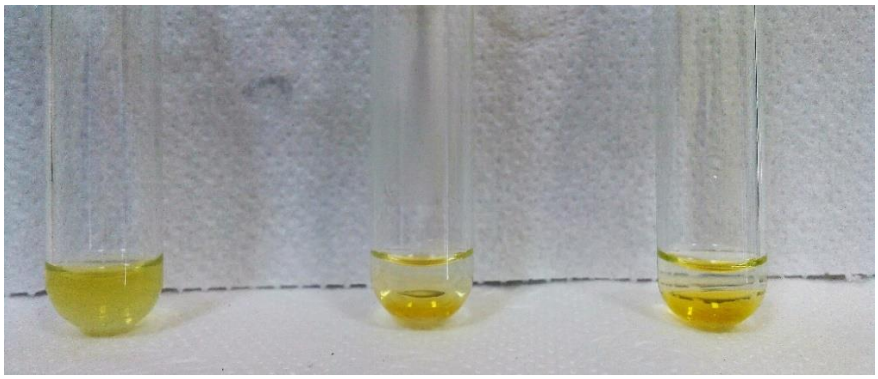


Figure 4.3. Triterpenoids detection in different parts of *Scolymus hispanicus*. From Left to right: Aerial parts, root barks and root internal parts.

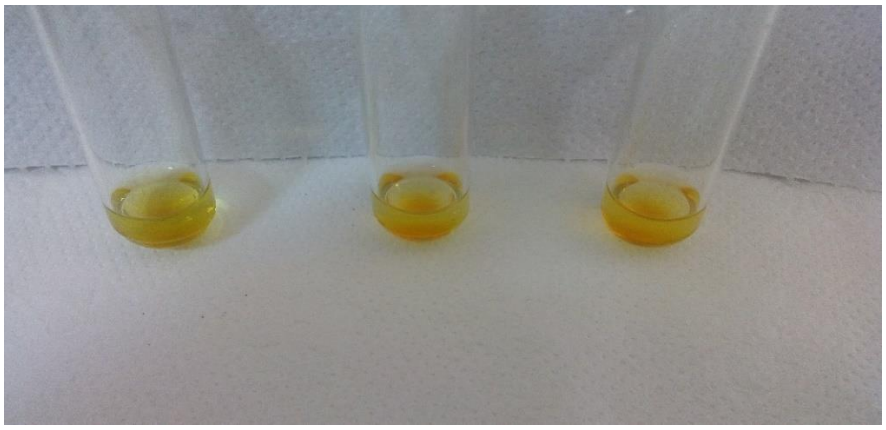


Figure 4.4. Alkaloid detection in different parts of *Scolymus hispanicus.* From left to right: Aerial parts, root barks and root internal parts.

4.1.5 Quantitative Analysis of Phytochemicals

4.1.5.1 Total Phenol Contents

Total phenol contents from all aerial parts of the five plant samples harvested and collected at different time periods throughout the year were determined by using standard procedures from the literature. There were no significant differences among the total phenol contents tested in different samples (Figure 4.5). Amongst all the samples tested, the samples from May gave highest amount of total phenol contents which were 5.1 mg gallic acid equivalent (GAE) in 100 mg of lyophilized extracts (LE) followed by July sample (4.4 mg), March sample (4.3 mg), November sample (4.1 mg) and January sample 3.4 mg. All the samples were tested in triplicate and the results were expressed as mg gallic acid equivalent (GAE) in 100 mg of lyophilized extracts. The total phenol contents from the root parts (root barks and root internal) and were also analyzed and when compared to the phenol amount obtained from aerial parts, it was almost five times less and due to non-appreciable amount, those results were not presented.

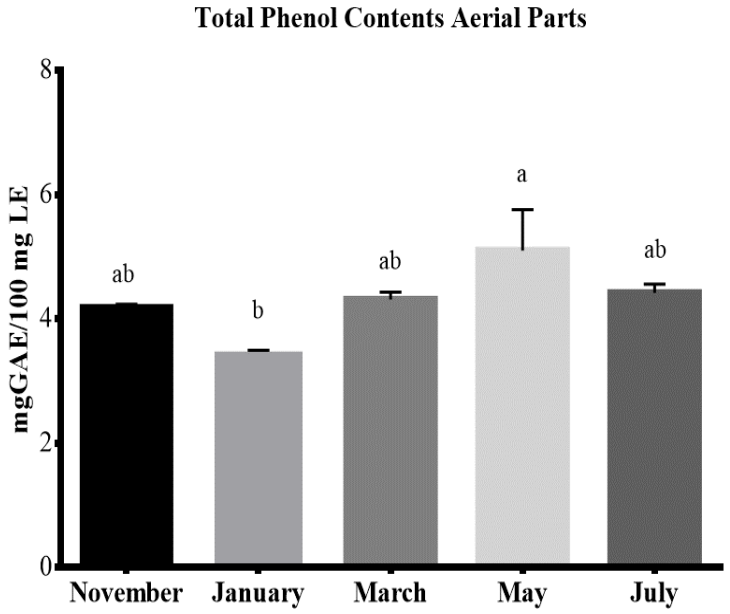


Figure: 4.5. Total phenol contents of the aerial parts of *Scolymus hispanicus* in different time periods. One-way Anova with Tukey post-test was used. Different letters indicate statistical differences between groups within each panel. (P)< 0.05 (at least). Bars depict mean ± SD; at least 3 measurments from each samples. (LE: Lyophilized Extracts)

4.1.5.2 Total Flavonoid Contents

Total flavonoid contents in the aerial parts of *S. hispanicus* were quantified. The data below shows the amount of total flavonoid contents found in samples harvested at different times periods (Figure 4.6). The samples from July gave highest amount of flavonoid contents which were 15.3 mg quercetin equivalent (QE) in 100 mg of lyophilized extracts (LE). November and January samples gave almost the same amount of flavonoids (14.3 and 14.0 mg QE/100 mg LE), followed by March sample 13.3 mg QE/100 mg LE and lastly the least amount of total flavonoid contents were given by May sample which was almost 10 mg QE/100 mg LE. Total flavonoid contents of the root parts were not in appreciable amounts when compared with the amount of total flavonoids obtained from the aerial parts, so that is why they are neglected.

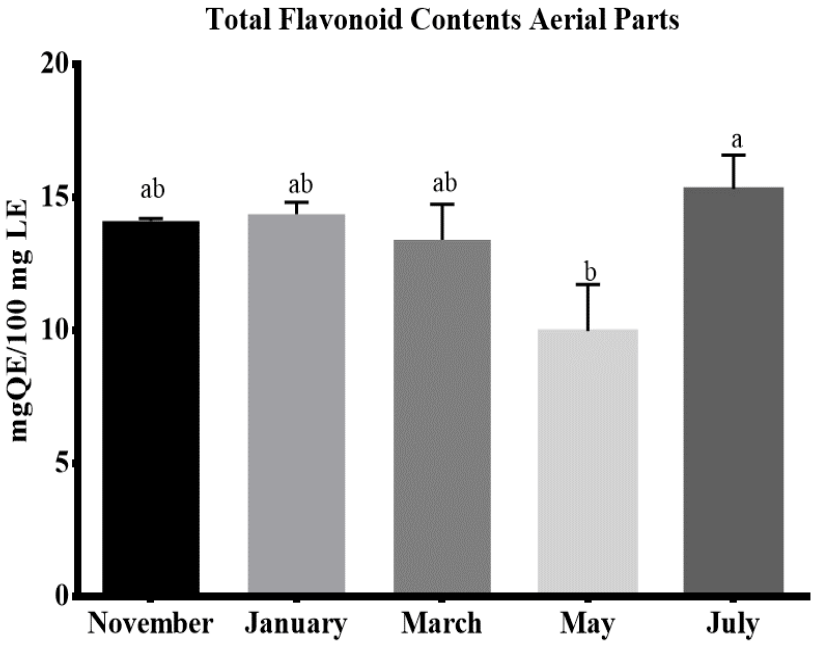


Figure 4.6. Total flavonoids contents of the aerial parts of *Scolymus hispanicus* in different time periods. One-way Anova with Tukey post-test was used. Different letters indicate statistical differences between groups within each panel. (P)< 0.05 (at least). Bars depict mean ± SD; at least 3 measurments from each samples. (LE: Lyophilized Extracts)

4.1.5.3 Total Tannin Contents

Total tannin contents from both aerial and root parts of *S. hispanicus* were analyzed. The aerial parts gave highest amount of tannins comparatively to the root parts of *S. hispanicus.* Total tannin contents were present in the root parts of *S. hispanicus.* As the amounts were extremely low almost 6 to 7 times lower than the aerial parts, the total tannin contents from the root parts were not presented. In the case of aerial parts, the November samples gave highest amount of total tannin contents which were 1.62 mg catechin equivalent (CE) in 100 mg of lyophilized extract (LE) (Figure 4.7). After November sample, the samples of March and January gave the highest amount of total tannins (1.37 and 1.13 mg CE/100 mg LE respectively). May and July samples also gave same but least amount of total tannins with 0.31 and 0.33 mg CE/100 mg LE values

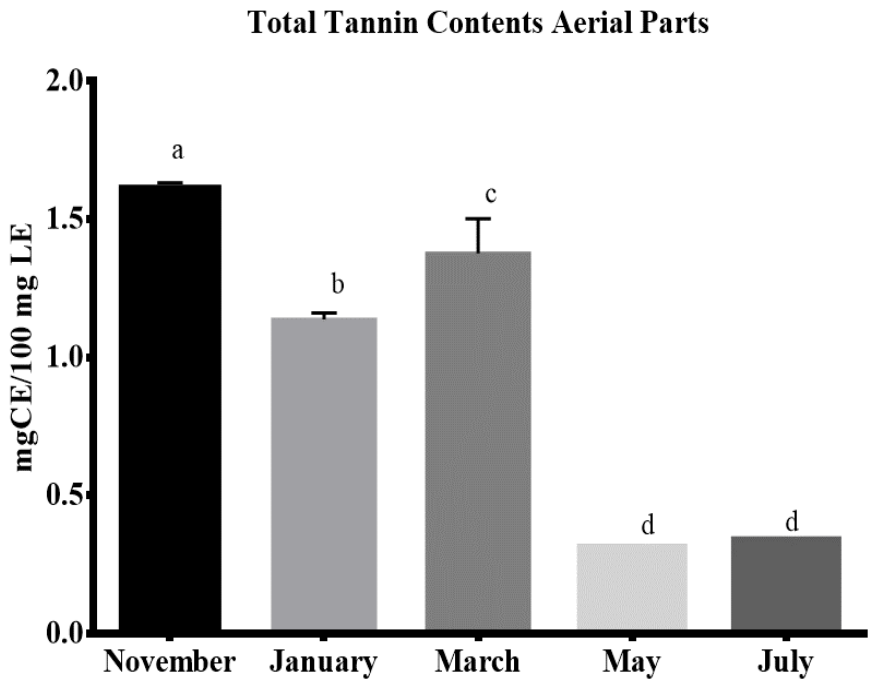
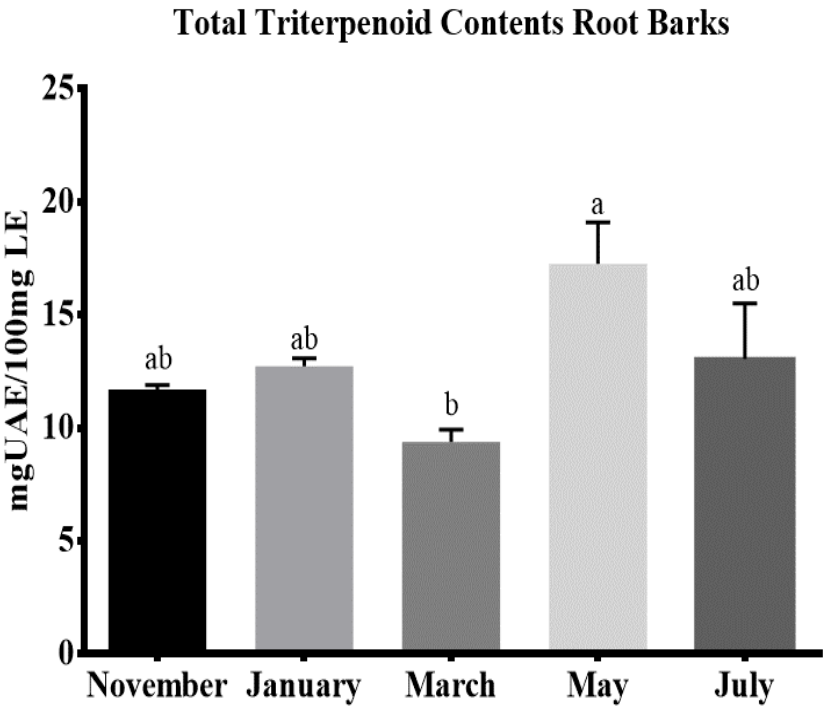


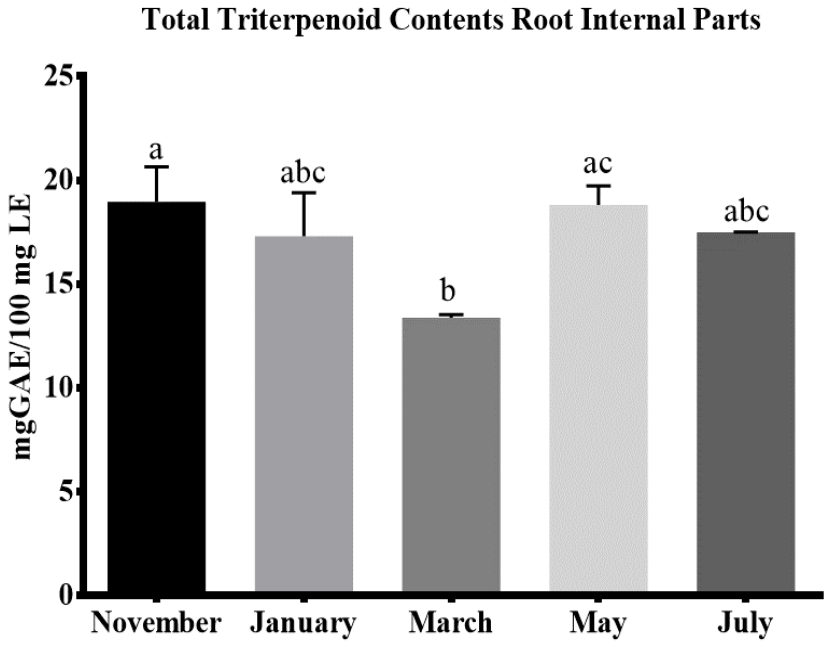
Figure 4.7. Total tannin contents of aerial parts of *Scolymus hispanicus* in different time periods. One-way Anova with Tukey post-test was used. Different letters indicate statistical differences between groups within each panel. (P)< 0.05 (at least). Bars depict mean ± SD; at least 3 measurments from each samples. (LE: Lyophilized Extracts).

4.1.5.4 Total Triterpenoid Contents

The root barks and root internal parts of *S. hispanicus* samples obtained during different time periods were analyzed for total triterpenoid contents. Interestingly both parts gave different results with respect to cultivation and harvesting time. In case of root barks, the samples collected from May gave highest amount of triterpenoids which was 17.25 mg ursolic acid equivalent (UAE) in 100 mg of lyophilized extract (LE). The lowest amount was obtained from March which was 9.36 mg UAE/100 mg LE (Figure 4.8a). In root internal parts, the samples from November gave highest amount of triterpenoid which was almost 19 mg UAE/100 mg LE and samples collected in March gave lowest amount of triterpenoids which were 13.3 mg UAE/100 mg LE (Figure 4.8b). So over all when calculated the root internal parts were richer in triterpenoid contents than the root barks. In order to check the availability of triterpenoids in the aerial parts, some analysis was carried out, however, the amount was comparatively very low than the root parts so the data was not presented. Complete data of total triterpenoid contents in the root barks and root internal parts of *S. hispanicus* from November, 2016 to July 2017 has been given in Figure 4.8a and b.



a)

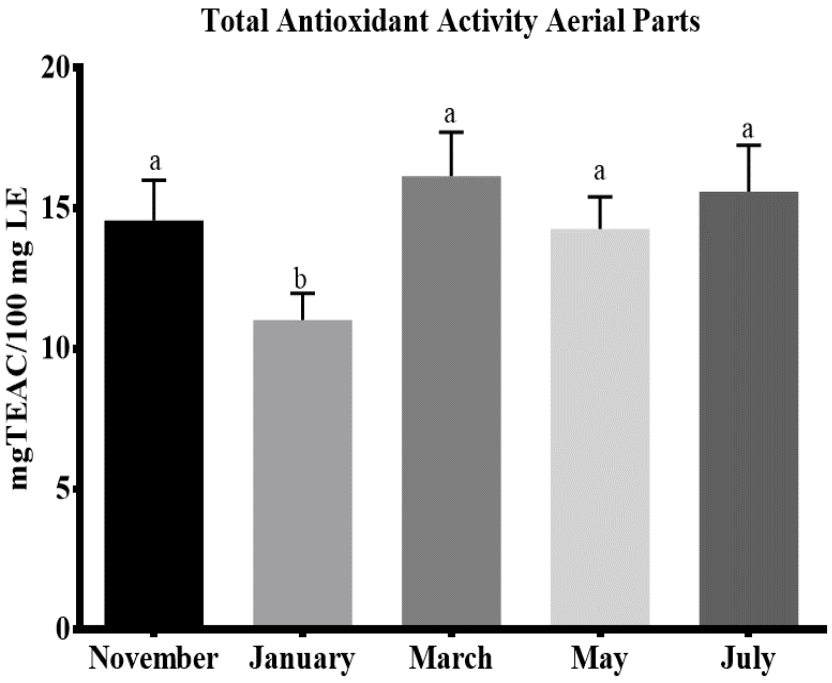


b)

Figure 4.8. a**)** Total triterpenoid contents from root barks of *Scolymus hispanicus* in different time periods. b) Total triterpenoid contents from root internal parts of *Scolymus hispanicus* in different time periods. One-way Anova with Tukey post-test was used. Different letters indicate statistical differences between groups within each panel. (P)< 0.05 (at least). Bars depict mean ± SD; at least 3 measurments from each sample. (LE: Lyophilized Extracts).

4.1.6 Total Antioxidant Activity

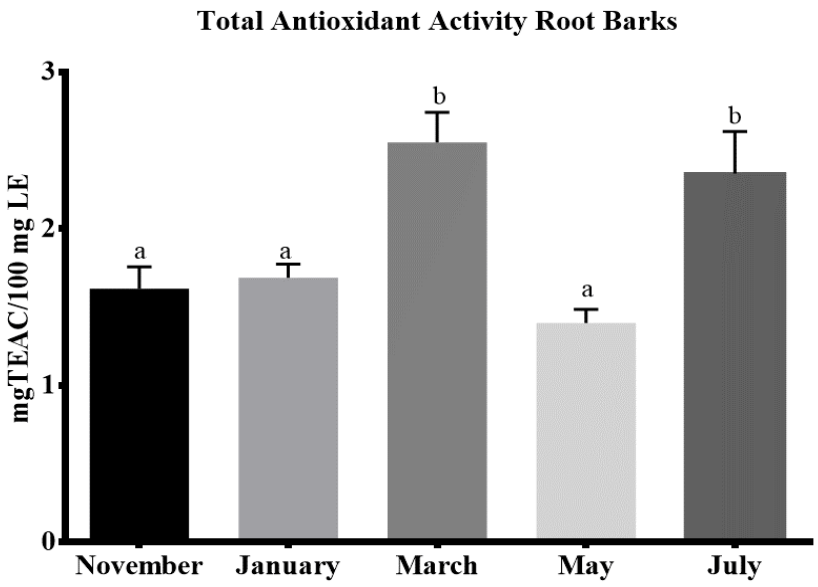
Total antioxidant activity was measured in all the extracts from *S. hispanicus* i.e. aerial parts, root barks, root internal parts and infusion extracts (Figure 4.9). Amongst all the parts analyzed, the aerial parts gave the highest amount of antioxidant activity. In case of root barks the samples from March and July gave highest antioxidant activity which were 2.5 and 2.3 mg TE/100 mg LE while in the case of root internal parts, samples from March, May and July gave highest antioxidant activity which were 2.7, 2.4, 2.3 mg TE/100 mg LE. All the data obtained has been explained in Figure 4.9a (aerial parts), 4.9b (root barks) and 4.9c (root internal parts). The antioxidant activity of the infusion extract of *S. hispanicus* was 0.12 mg trolox equivalent/ml.



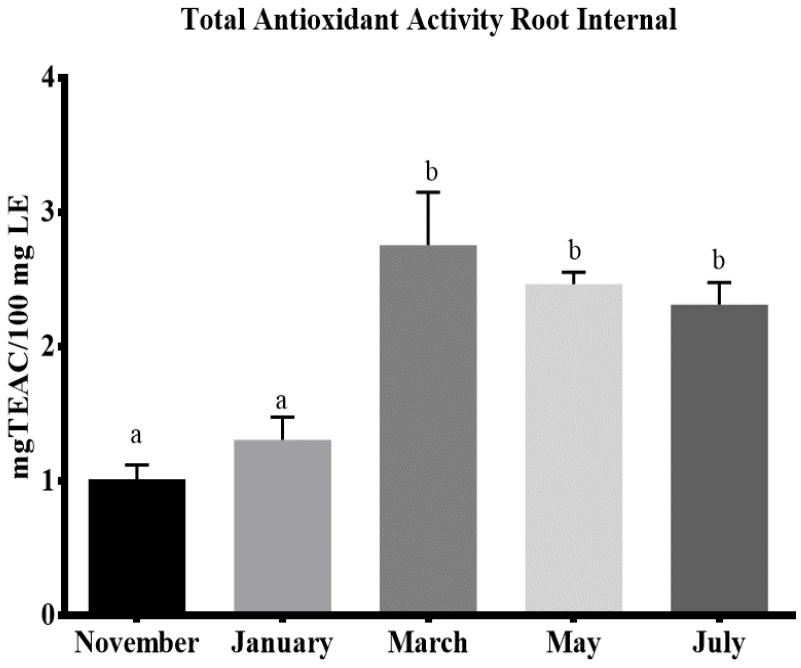
a)

Figure 4.9. a) Total antioxidant activity from aerial parts of *Scolymus hispanicus* in different time periods. b) Total antioxidant activity from root barks of *Scolymus hispanicus* in different time periods. c) Total antioxidant activity from root internal parts of *Scolymus hispanicus* in different time periods.One-way Anova with Tukey post-test was used. Different letters indicate statistical differences between groups within each panel (P)< 0.05 (at least). Bars depict mean ± SD; at least 3 measurments from each samples. (LE: Lyophilized Extracts).

(Cont. on next page)



b)



c)

Figure 4.9 (Cont.)

4.1.7 Thin Layer Chromatography (TLC) Analysis

Thin Layer Chromatography (TLC) analysis was done to analyze some of the phytochemicals in the lyophilized crude extracts of *S. hispanicus.* TLC is basically a technique which is used for the separation of the mixture into its separate components. Firstly, a non polar system was developed by using hexane and ethyl acetate (8:2). Standard triterpenoid compounds such as, taraxasterol, taraxasterol acetate and ursolic acid after dissolving in chloroform individually, were spotted on the TLC plate and then dipped into the solvent system in the TLC tank. After the completion of the process when visualized under UV light, clear bands of the standard compounds were seen under UV light (Figure 4.10). Later on, the lyophilized extracts from all the three parts (aerial parts, root barks and root internal parts) of *S. hispanicus* were also dissolved in chloroform and spotted on the TLC plate along with the standard triterpenoid compounds (taraxasterol, taraxasterol acetate and ursolic acid). This time again clear bands were seen of the standard triterpenoid compounds but there were no bands of the lyophilized extracts of *S. hispanicus*, which indicated the absence of free triterpenoid compounds in the crude extracts of *S. hispanicus.*

In order to check the presence or absence of the glycosidic compounds in the crude extracts of *S. hispanicus* another solvent system was developed which was more polar as compared to the previously used solvent system. In this case, chloroform, methanol and water (61:32:7) were used as solvent in the TLC tank. The solutions of the lyophilized extracts of *S. hispanicus* in chloroform were spotted on the TLC plate and dipped into the newly developed and comparatively polar solvent system. After few minutes when the TLC plate was taken out and visualized under UV light then clear bands were seen (Figure 4.11) of the all the three lyophilized crude extracts (aerial parts, root barks and root internal parts) of *S. hispanicus*, which indicated the presence of the glycosidic compounds in these extracts of *S. hispanicus*. It was probable that the glycosidic compounds were triterpenoid sponins.

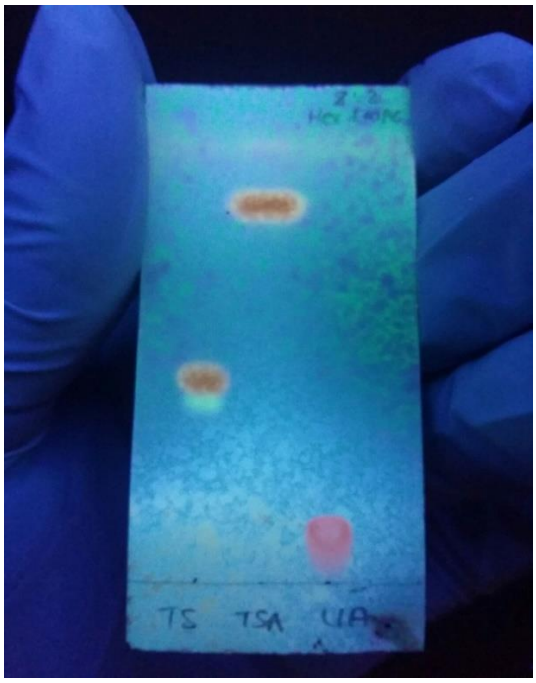


Figure 4.10. TLC plate with bands of taraxasterol, taraxasterol acetate and ursolic acid. TS: Taraxasterol. TSA: Taraxasterol Acetate. UA: Ursolic acid.

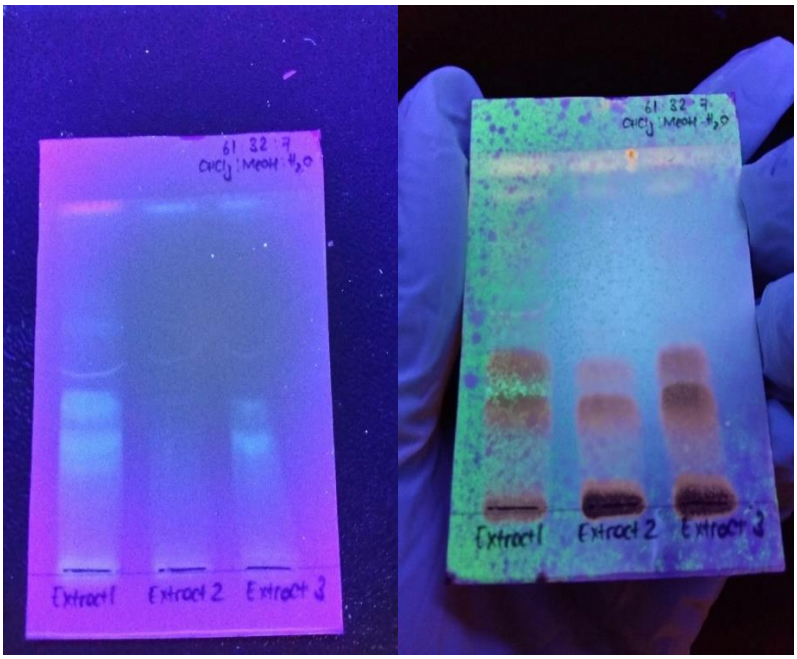
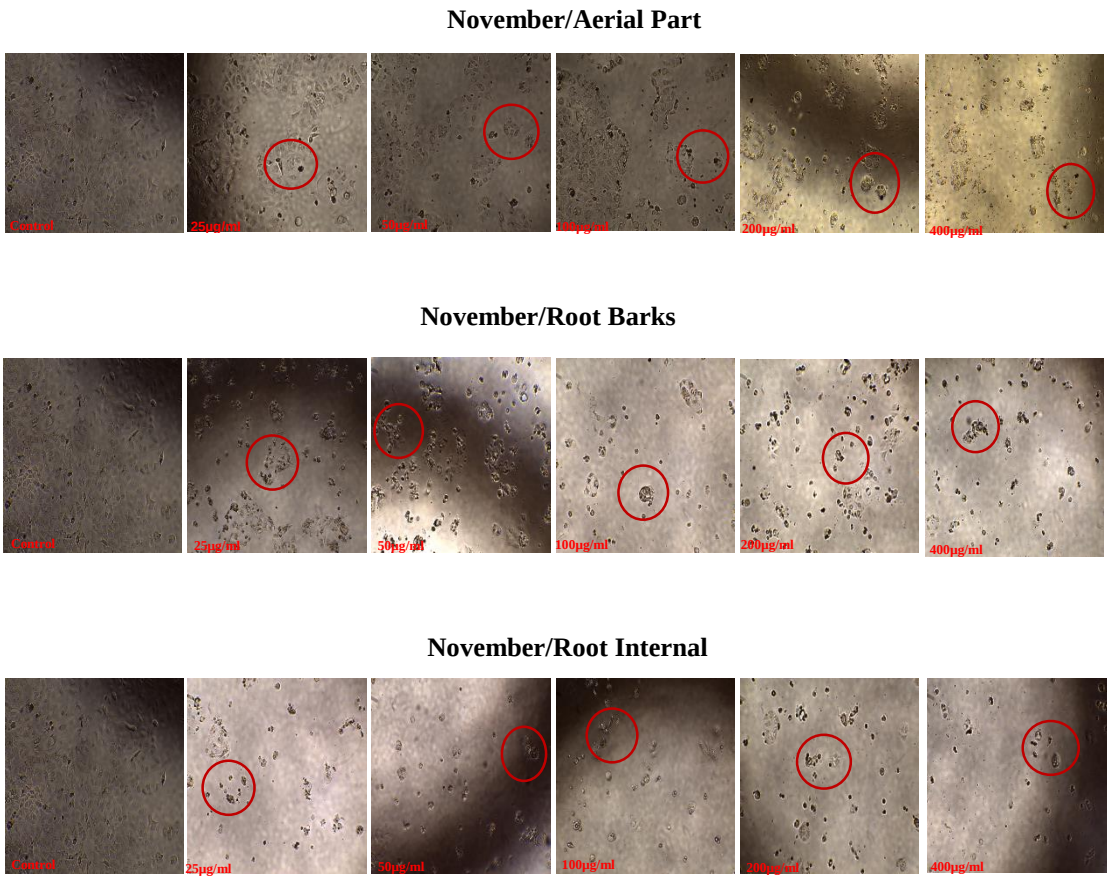


Figure 4.11. TLC plates with clear bands from all the three parts (aerial parts, root barks and root internal parts) of *S. hispanicus*. Extract 1. Aerial Parts. Extract 2. Root Barks. Extract 3. Root Internal Parts.

4.1.8 Effect of crude extract on Caco-2 cells

After applying and incubation with different concentrations of the crude extracts of *S. hispanicus* for almost 48 h, the crude extracts of *S. hispanicus* showed cytotoxic effects on Caco-2 cells growth (Figure 10). There were also less number of cells observed in the cell containing wells treated with the crude extracts compared to non-teated cells. It was also observed that, the cytotoxic effects were crude extracts cncentration dependent.

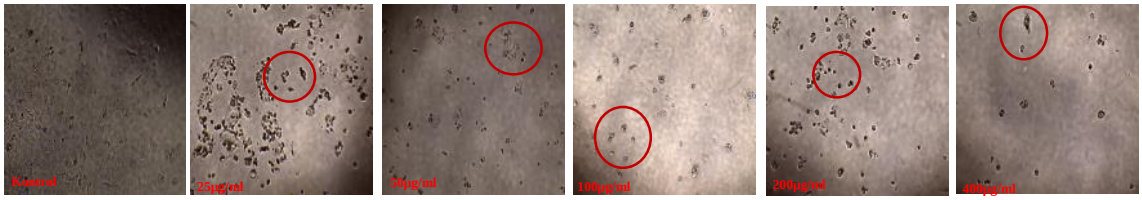


a)

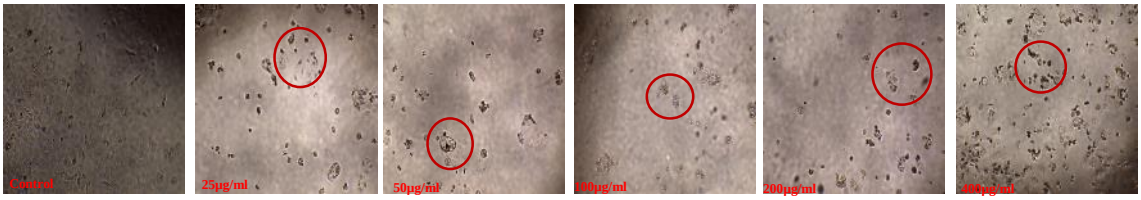
Figure 12. Cytotoxic effects of the crude extracts of *S. hispanicus* on Caco-2 cells after 48 h of incubation with the crude extracts. a) November sample. b) May sample.

(Cont. on next page)

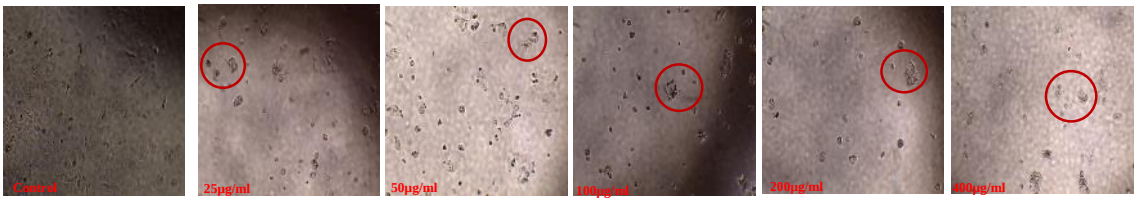
May/Aerial Parts



May/ Root Barks



May/ Root Internal



b)

Figure 4.12 (Cont.)

4.2 Discussion

Scolymus hispanicus is grown almost in every region of Turkey, but due to the limited use of this plant, its economic value is not high (Altiner and Sahan, 2016). In an effort to add value to this plant, the chemical composition and characterization of some of the major bioactive compounds and antioxidant activities of the crude extracts of *S. hispanicus* were investigated in this study. Previous agricultural research has shown that *S. hispanicus* can be cultivated (Dr. Ünal Karık, personal communication). It has also shown that the harvest time was not limited to a few months but can be harvested almost all along the year. Therefore, one of the aims in this study was the assessment of the effect of harvesting time on the composition, hence on the health benefits of the plant. There are three main parts of *S. hispanicus* regarding the agriculture and usage of it. Aerial part is the part above the soil composed of the stems and the leaves. The root has a soft bark, which is the edible part used to prepare food, while the internal part of the root is hard and not suitable for food preparation. That is why, in this study these parts were tested separately and the results were compared.

Generally, the effect of the harvesting time varied regarding the phytochemicals. The concentrations of some of them did not change significantly with time of harvest, while the effect was noticeable in some others. Generally, it is harvested for eating purposes in the month of May in Izmir province and its surrounding areas. A very few reports are available regarding the different bioactive compounds present in this plant and no reports have been published for the determination of different phytochemicals in *S. hispanicus* cultivated and harvested during different time periods. So, for the first time *S. hispanicus* was analyzed for different phytochemicals and antioxidant activities. The amount of phytochemicals was different in each plant sample grown and harvested during different months of the year. Total phenols contents were higher in the aerial parts as compared to the root parts. In the case of the aerial parts, the phenolic contents were higher in the sample obtained in the month of May followed by July, March and November samples. The least amounts of total phenol contents were observed in January samples. It may be due to the effect of light intensity, because light intensity also induces the biosynthesis of phenolic compounds (Harbowy and Balentine., 1997). As days become longer from March to July, so it can be said that the amount of non-volatile secondary metabolites maybe higher in these months. In one report by Gadzovska et al., (2013) same results were also observed in the case of *Hypericum*

perforatum which was cultivated in both winter and summer to analyze the seasonal effects on the bioactive constituents. In that report, it was observed that the synthesis of bioactive constituents such as hypericin and pseudohypericin was increased in summer and decreased in winter. Similarly, other factors such as temperature also affect their biosynthesis and activities. The amount of total phenolic contents also increases in water stress, because they are known to hydrolyze the glycosides under such situations (Deshmukh., 2010). In one other report by Phrompittayarat et al., (2011) in *Bacopa monneiri* the bioactive compounds were found high in the months from July to October (monsoon season) and lowest from March to June. The reason for these differences were explained as different temperature, water conditions and stress which effected the inside contents. As in monsoon period, the availability of water, sunlight and other optimum conditions are more easily available as compared to other months so this was the reason of high yield of internal contents biosynthesis in *B. monneri*. Rise and fall along with other variations in seasons effect the availability of the bioactive compounds in medicinal plants and hence also influence the therapeutic effect of the plants. The antioxidant activity is correlated with the amount of total phenolic contents (Soni et al., 2015). In this study, the overall total phenolic contents of *S. hispanicus* were found to be higher in the aerial parts than the root parts. So when analyzed for the total antioxidant activity the aerial parts of *S. hispanicus* had highest antioxidant activities, followed by root internal parts and root barks. The significantly high antioxidant activities in the aerial parts can be attributed to the higher amount of total phenolic contents in the aerial parts as compared to the root parts. Besides that, there were also differences in between the antioxidant activities of individual samples from different months, which showed that seasonal variations may have effected the chemicals composition of *S. hispanicus.* In case of aerial parts, the plant samples grown in the months of March, May, July and November had significantly higher antioxidant activities than the samples grown in January, which indicated that spring and summer are most probably the best seasons for higher quantities of the bioactive compounds. Thus, based on the findings in the literature, it can be said that the amount of total bioactive constituents found in *S. hispanicus* harvested and cultivated during different months of the year may be affected by the different environmental conditions such as water, stress, temperature, light intensity and light duration and others.

The amount of total triterpenoids were higher in the roots than the aerial parts. The seasonal variations also effected the contents of total triterpenoids in the root parts

and they showed the same pattern like total phenol contents. Over all, the highest amount of total triterpenoids were observed in the samples harvested in May. Two parts from the root (root barks and root internal) were analyzed separately. The root internal parts gave higher amount of total triterpenoids as compared to the root barks. Root barks are usually consumed as food and the root internal parts are considered as waste residue. So that, for the first time based on our knowledge, the root internal parts were analyzed for the determination of total triterpenoids of *S. hispanicus,* which can add economic value to this plant and can be industrialized for the production of licensed medicines. Already in the past one licensed syrup by the name of Lityazol Cemil was developed from the root extracts of *S. hispanicus.* That syrup had been used for the removal of kidney stones but the production stoped due to lack of raw material. It was believed that the kidney stone removing effects of Lityazol Cemil were due to taraxasteryl acetate which is a triterpenoid (Tunalier et al., 1997). In TLC analysis it was observed, that there were no free triterpenoids in *S. hispanicus,* such as taraxasterol, taraxasterol acetate and ursolic acid but some of the glycosidic compounds such as triterpenoid saponins may be present in *S. hispanicus*. There are reports in the literature showing that, triterpenoids such taraxasteryl acetate are present in *S. hispanicus*, but based on our TLC analysis there were no triterpenoids. So it can be consider that either the triterpenoids may be in very low quantity and might not be extracted by the extraction procedures which we used or they maybe present in conjugation with other compounds such as glycosides. So for this reason, it is suggested that further research, such as new extraction techniques with different and most suitable conditions must be carried out to get the full picture of triterpenoids and other bioactive compounds present in *S. hispanicus*. This will also add credits to the importance and economic values of *S. hispanicus* as well.

The amount of total phenol contents is generally higher in the leaves than in any other parts of the plant (Raya et al., 2015). So the results obtained from *S. hispanicus* were consistent with the report published by Raya et al., (2015). Similarly, the amount of phytochemicals is also affected by physiology of the plant and duration of storage. Younger plants most often contain the higher amount of phytochemicals in comparison to the older plants (Raya et al., 2015). The best season for the cultivation of *S. hispanicus* and its use as a food is spring season, so at that time the plant is younger and a little bigger in size and this may be the reason that it contains high amount of phenolics, triterpenoids and high antioxidant activities in the plant samples cultivated

and harvested in the spring season. As *S. hispanicus* is grown throughout the year so, it is suggested that further research must be done for the determination of other bioactive compounds from those parts of this plant which are usually considered as waste such as the aerial parts and root internal parts. In this way, *S. hispanicus* will add values to the markets of natural medicine production and may help to produce new, easily available and cheap drugs.

CHAPTER 5

CONCLUSION

This study was conducted to analyze the total contents of some of the bioactive compounds from *Scolymus hispanicus* which is known to have benefical health effects. This plant generally consumed as vegetable in Turkey, specially in Izmir province. Its root barks are eaten and root internal and aerial parts are considered as residues. According to some of the ethnobotanical studies, it was widely used for the treatment of various diseases by the local folks in the past. In our study *S. hispanicus,* cultivated and harvested during different time periods, were analyzed for different bioactive compounds, antioxidant activities and cytotoxic effects. There were differences amongst the different phytochemicals from different samples of *S. hispanicus* cultivated and harvested in different time periods. The polyphenol contents were higher in the aerial parts as compared to the root parts (root barks and root internal parts). Similarly the total triterpenoid contents were higher in the root parts (root barks and root internal parts). Environmental factors and seasonal variations such as intensity of light, duration of the day, availability of the water, different stressful conditions along with others may have also effected the biosynthesis of these bioactive compounds.

The environmental factors also effect the pharmacological activities of the plants. So, when analyzed for the antioxidant activities then comparitavely the aerial parts showed the higher antioxidant activities than the root parts (root barks and root internal parts). There were also some small scale differences in the antioxidant activities of the aerial parts, root barks and root internal parts of different samples cultivated and harvested at different time periods. In case of root parts, the root internal parts showed higher antioxidant activities than root barks. The TLC analysis showed the absence of free triterpenoids such as taraxasterol, taraxatserol acetate and ursolic acid but there maybe glycosidic compounds in *S. hispanicus* such as triterpenoid saponnins, which further may be analyzed by using different extraction and quantification procedures.

Based on the knowledge from previous ethnobotanical studies, to check the beneficial health effects of *S. hispanicus*, its crude extracts were applied to the colon cancer cell lines (Caco-2). Caco-2 cell lines were treated with the crude extracts of *S. hispanicus* from all the parts (aerial parts, root barks, root internal parts). After 48 h of

incubation with the crude extracts, cytotoxic effects of the crude extracts of *S. hispanicus* were observed when compared to the negative controls. It might be an indication of the cytotoxic and preventative effects of the crude extracts of *S. hispanicus* on colon cancer. It was also observed that the effects of crude extracts were concentration dependent.

So, frurther research should be done to analyze specific bioactive compounds of *S.hispanicus* to evaluate cytotoxic effect on cancer cell growth. It is also important to show whether bioactive compounds are selecetive or nonselective for colon cancer preventation. It may help in the discovery of new, cheap and easily available drugs such as Lityazol Cemil from *S. hispanicus.*

REFERENCES

Aherne, S. A., & O'Brien, N. M. (1999). Protection by the flavonoids myricetin, quercetin, and rutin against hydrogen peroxide-induced DNA damage in Caco-2 and Hep G2 cells. *Nutrition and cancer*, *34*(2), 160-166.

Altiner, D. D., & Sahan, Y. (2016). A Functional Food Additive: Scolymus Hispanicus L. Flour. *International Journal of Food Engineering*, *2*(2).

Amarowicz, R., Pegg, R. B., Rahimi-Moghaddam, P., Barl, B., & Weil, J. A. (2004). Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. *Food chemistry*, *84*(4), 551-562.

Anantharaju, P. G., Gowda, P. C., Vimalambike, M. G., & Madhunapantula, S. V. (2016). An overview on the role of dietary phenolics for the treatment of cancers. *Nutrition journal*, *15*(1), 99.

Araújo, J. R., Gonçalves, P., & Martel, F. (2011). Chemopreventive effect of dietary polyphenols in colorectal cancer cell lines. *Nutrition Research*, *31*(2), 77-87.

Babu, P. V. A., Liu, D., & Gilbert, E. R. (2013). Recent advances in understanding the anti-diabetic actions of dietary flavonoids. *The Journal of nutritional biochemistry*, *24*(11), 1777-1789.

Balasundram, N., Sundram, K., & Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food chemistry*, *99*(1), 191-203.

Bandopadhyaya, S., Ramakrishnan, M., Thylur, R. P., & Shivanna, Y. (2015). In-vitro evaluation of plant extracts against colorectal cancer using HCT 116 cell line. *Int J Plant Sci Ecol*, *1*(3), 107-12.

Behzad, S., Ebrahim, K., Mosaddegh, M., & Haeri, A. (2016). Primula auriculata Extracts Exert Cytotoxic and Apoptotic Effects against HT-29 Human Colon Adenocarcinoma Cells. *Iranian journal of pharmaceutical research: IJPR*, *15*(1), 311.

Benítez, G., González-Tejero, M. R., & Molero-Mesa, J. (2010). Pharmaceutical ethnobotany in the western part of Granada province (southern Spain): ethnopharmacological synthesis. *Journal of Ethnopharmacology*, *129*(1), 87-105.

Bermejo, J. E. H., & León, J. (Eds.). (1994). *Neglected crops: 1492 from a different perspective* (No. 26). Food & Agriculture Org.

Bishayee, A., Ahmed, S., Brankov, N., & Perloff, M. (2011). Triterpenoids as potential agents for the chemoprevention and therapy of breast cancer. *Frontiers in bioscience: a journal and virtual library*, *16*, 980.

Bnouham, M., Mekhfi, H., Legssyer, A., & Ziyyat, A. (2002). Ethnopharmacology Forum Medicinal plants used in the treatment of diabetes in Morocco. *International Journal of Diabetes & Metabolism*, *10*, 33-50.

Briskin, D. P. (2000). Medicinal plants and phytomedicines. Linking plant biochemistry and physiology to human health. *Plant physiology*, *124*(2), 507-514.

Caro, I., Boulenc, X., Rousset, M., Meunier, V., Bourrié, M., Julian, B., ... & Fabre, G. (1995). Characterisation of a newly isolated Caco-2 clone (TC-7), as a model of transport processes and biotransformation of drugs. *International journal of pharmaceutics*, *116*(2), 147-158.

Chantret, I., Barbat, A., Dussaulx, E., Brattain, M. G., & Zweibaum, A. (1988). Epithelial polarity, villin expression, and enterocytic differentiation of cultured human colon carcinoma cells: a survey of twenty cell lines. *Cancer research*, *48*(7), 1936-1942.

Cheynier, V. (2005). Polyphenols in foods are more complex than often thought. *The American journal of clinical nutrition*, *81*(1), 223S-229S.

Chitme, H. R., Chandra, R., & Kaushik, S. (2004). Studies on anti-diarrhoeal activity of Calotropis gigantea R. Br. in experimental animals. *J Pharm Pharm Sci*, *7*(1), 70-75.

Chlopčíková, Š., Psotová, J., Miketová, P., & Šimánek, V. (2004). Chemoprotective effect of plant phenolics against anthracycline-induced toxicity on rat cardiomyocytes. Part I. Silymarin and its flavonolignans. *Phytotherapy Research*, *18*(2), 107-110.

Chung, K. T., Wong, T. Y., Wei, C. I., Huang, Y. W., & Lin, Y. (1998). Tannins and human health: a review. *Critical reviews in food science and nutrition*, *38*(6), 421-464.

Deshmukh, R. N. (2012). Osmolyte accumulation in Sorghum bicolor under water stress. *Bionano frontier*, *5*(2), 204-09.

Doughari, J. H. (2012). Phytochemicals: extraction methods, basic structures and mode of action as potential chemotherapeutic agents. In *Phytochemicals-A global perspective of their role in nutrition and health*. InTech.

Dzubak, P., Hajduch, M., Vydra, D., Hustova, A., Kvasnica, M., Biedermann, D., ... & Sarek, J. (2006). Pharmacological activities of natural triterpenoids and their therapeutic implications. *Natural product reports*, *23*(3), 394-411.

El Kar, C., Ferchichi, A., Attia, F., & Bouajila, J. (2011). Pomegranate (Punica granatum) juices: chemical composition, micronutrient cations, and antioxidant capacity. *Journal of Food Science*, *76*(6), C795-C800.

Fukumitsu, S., Villareal, M. O., Fujitsuka, T., Aida, K., & Isoda, H. (2016). Anti-inflammatory and anti-arthritic effects of pentacyclic triterpenoids maslinic acid through NF-κB inactivation. *Molecular nutrition & food research*, *60*(2), 399-409.

Gadzovska, S., Maury, S., Delaunay, A., Spasenoski, M., Hagège, D., Courtois, D., & Joseph, C. (2013). The influence of salicylic acid elicitation of shoots, callus, and cell suspension cultures on production of naphtodianthrones and phenylpropanoids in Hypericum perforatum L. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *113*(1), 25-39.

Gan, L. S. L., & Thakker, D. R. (1997). Applications of the Caco-2 model in the design and development of orally active drugs: elucidation of biochemical and physical barriers posed by the intestinal epithelium. *Advanced Drug Delivery Reviews*, *23*(1-3), 77-98.

Ghosh, D. (2015). Tannins from foods to combat diseases. *International Journal Pharmaceutical Research Rev*, *4*(5), 40-44.

Giada, M. D. L. R. (2013). Food phenolic compounds: main classes, sources and their antioxidant power. In *Oxidative stress and chronic degenerative diseases-A role for antioxidants*. InTech.

Godghate, A. G., & SawanT, R. S. (2014). Phytochemical analysis of leaves of tectona Grandis linn. *International journal of pharma and biosciences*, *5(1)*, 355-359.

Gonçalves, P., Araújo, J. R., Pinho, M. J., & Martel, F. (2011). In vitro studies on the inhibition of colon cancer by butyrate and polyphenolic compounds. *Nutrition and cancer*, *63*(2), 282-294.

Grey, C., Widén, C., Adlercreutz, P., Rumpunen, K., & Duan, R. D. (2010). Antiproliferative effects of sea buckthorn (Hippophae rhamnoides L.) extracts on human colon and liver cancer cell lines. *Food chemistry*, *120*(4), 1004-1010.

Gupta, Monika., Thakur, Shweta., Sharma, Anuradha., & Gupta, sudhakar. (2013). Qualitative and quantitative analysis of phytochemicals and pharmacological value of some dye yielding medicinal plants. *Oriental Journal of chemistry*, *29*(2), 475-481.

Haggar, F. A., & Boushey, R. P. (2009). Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors. *Clinics in colon and rectal surgery*, *22*(04), 191-197.

Han, N., & Bakovic, M. (2015). Biologically active triterpenoids and their cardioprotective and anti-inflammatory effects. *Journal of Bioanalysis and Biomedicine S*, *12*(005), 1948-5.

Harbowy, M. E., Balentine, D. A., Davies, A. P., & Cai, Y. (1997). Tea chemistry. *Critical reviews in plant sciences*, *16*(5), 415-480.

Heim, K. E., Tagliaferro, A. R., & Bobilya, D. J. (2002). Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *The Journal of nutritional biochemistry*, *13*(10), 572-584.

Hidalgo, I. J., Raub, T. J., & Borchardt, R. T. (1989). Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology*, *96*(3), 736-749.

Hill, R. A., & Connolly, J. D. (2015). Triterpenoids. *Natural product reports*, *32*(2), 273-327.

Hodgson, J. M. (2008). Tea flavonoids and cardiovascular disease. *Asia Pacific Journal of clinical nutrition*, *17*(S1), 288-290.

Hung, D., Ou, B., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assay. *J. Agric. Food Chem*, *53*, 1841-1856.

Indap, M. A., Radhika, S., Motiwale, L., & Rao, K. V. K. (2006). Anticancer activity of phenolic antioxidants against breast cancer cells and a spontaneous mammary tumor. *Indian Journal of Pharmaceutical Sciences*, *68*(4), 470-474.

Iqbal, E., Salim, K. A., & Lim, L. B. (2015). Phytochemical screening, total phenolics and antioxidant activities of bark and leaf extracts of Goniothalamus velutinus (Airy Shaw) from Brunei Darussalam. *Journal of King Saud University-Science*, *27*(3), 224-232.

Jamshieed, S., Das, S., Sharma, M. P., & Srivastava, P. S. (2010). Difference in in vitro response and esculin content in two populations of Taraxacum officinale Weber. *Physiology and Molecular Biology of Plants*, *16*(4), 353-358.

Kapiszewska, M., Soltys, E., Visioli, F., Cierniak, A., & Zajac, G. (2005). The protective ability of the Mediterranean plant extracts against the oxidative DNA damage. The role of the radical oxygen species and the polyphenol content. *Journal of Physiology and Pharmacology. Supplement*, *56*(1), 183-197.

Khan, A. M., Qureshi, R. A., Ullah, F., Gilani, S. A., Nosheen, A., Sahreen, S., ... & Murad, W. (2011). Phytochemical analysis of selected medicinal plants of Margalla Hills and surroundings. *Journal of Medicinal Plants Research*, *5*(25), 6055-6060.

Khuhaprema, T., & Srivatanakul, P. (2008). Colon and rectum cancer in Thailand: an overview. *Japanese journal of clinical oncology*, *38*(4), 237-243.

Kim, H. S. (2005). Do not put too much value on conventional medicines. *Journal of ethnopharmacology*, *100*(1), 37-39.

Kumar, S., & Pandey, A. K. (2013). Chemistry and biological activities of flavonoids: an overview. *The Scientific World Journal*, *2013*.

Kuo, S. M. (1996). Antiproliferative potency of structurally distinct dietary flavonoids on human colon cancer cells. *Cancer letters*, *110*(1-2), 41-48.

Lesellier, E., Destandau, E., Grigoras, C., Fougère, L., & Elfakir, C. (2012). Fast separation of triterpenoids by supercritical fluid chromatography/evaporative light scattering detector. *Journal of Chromatography A*, *1268*, 157-165.

Meunier, V., Bourrie, M., Berger, Y., & Fabre, G. (1995). The human intestinal epithelial cell line Caco-2; pharmacological and pharmacokinetic applications. *Cell biology and toxicology*, *11*(3-4), 187-194.

Miller, N. J., & Rice-Evans, C. A. (1997). Factors influencing the antioxidant activity determined by the ABTS•+ radical cation assay. *Free radical research*, *26*(3), 195-199.

Morales, P., Ferreira, I. C., Carvalho, A. M., Sánchez-Mata, M. C., Cámara, M., Fernández-Ruiz, V., & Tardío, J. (2014). Mediterranean non-cultivated vegetables as dietary sources of compounds with antioxidant and biological activity. *LWT-Food Science and Technology*, *55*(1), 389-396.

Moyle, C. W., Cerezo, A. B., Winterbone, M. S., Hollands, W. J., Alexeev, Y., Needs, P. W., & Kroon, P. A. (2015). Potent inhibition of VEGFR-2 activation by tight binding of green tea epigallocatechin gallate and apple procyanidins to VEGF: Relevance to angiogenesis. *Molecular nutrition & food research*, *59*(3), 401-412.

Ozkan, G., Kamiloglu, S., Ozdal, T., Boyacioglu, D., & Capanoglu, E. (2016). Potential use of turkish medicinal plants in the treatment of various diseases. *Molecules*, *21*(3), 257.

Pacheco-Palencia, L. A., Noratto, G., Hingorani, L., Talcott, S. T., & Mertens-Talcott, S. U. (2008). Protective effects of standardized pomegranate (Punica granatum L.) polyphenolic extract in ultraviolet-irradiated human skin fibroblasts. *Journal of agricultural and food chemistry*, *56*(18), 8434-8441.

Palombo, E. A. (2006). Phytochemicals from traditional medicinal plants used in the treatment of diarrhoea: modes of action and effects on intestinal function. *Phytotherapy Research*, *20*(9), 717-724.

Pampaloni, B., Palmini, G., Mavilia, C., Zonefrati, R., Tanini, A., & Brandi, M. L. (2014). In vitro effects of polyphenols on colorectal cancer cells. *World journal of gastrointestinal oncology*, *6*(8), 289.

Pandey, K. B., & Rizvi, S. I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative medicine and cellular longevity*, *2*(5), 270-278.

Patil, K. R., Mohapatra, P., Patel, H. M., Goyal, S. N., Ojha, S., Kundu, C. N., & Patil, C. R. (2015). Pentacyclic triterpenoids inhibit IKKβ mediated activation of NF-κB pathway: in silico and in vitro evidences. *PloS one*, *10*(5), e0125709.

Pereira, D. M., Valentão, P., Pereira, J. A., & Andrade, P. B. (2009). Phenolics: From chemistry to biology.

Phrompittayarat, W., Jetiyanon, K., Wittaya-areekul, S., Putalun, W., Tanaka, H., Khan, I., & Ingkaninan, K. (2011). Influence of seasons, different plant parts, and plant growth stages on saponin quantity and distribution in Bacopa monnieri. *Songklanakarin Journal of Science & Technology*, *33*(2).

Pitchakarn, P., Suzuki, S., Ogawa, K., Pompimon, W., Takahashi, S., Asamoto, M., ... & Shirai, T. (2011). Induction of G1 arrest and apoptosis in androgen-dependent human prostate cancer by Kuguacin J, a triterpenoid from Momordica charantia leaf. *Cancer letters*, *306*(2), 142-150.

Polo, S., Tardío, J., Vélez-del-Burgo, A., Molina, M., & Pardo-de-Santayana, M. (2009). Knowledge, use and ecology of golden thistle (Scolymus hispanicus L.) in Central Spain. *Journal of ethnobiology and ethnomedicine*, *5*(1), 42.

Ramos, A. A., Pereira-Wilson, C., & Collins, A. R. (2010). Protective effects of ursolic acid and luteolin against oxidative DNA damage include enhancement of DNA repair in Caco-2 cells. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *692*(1), 6-11.

Rasool Hassan, B. A. (2012). Medicinal plants (importance and uses). *Pharmaceut Anal Acta*, *3*, e139.

Raya, K. B., Ahmad, S. H., Farhana, S. F., Mohammad, M., Tajidin, N. E., & Parvez, A. (2015). Changes in phytochemical contents in different parts of Clinacanthus nutans (Burm. f.) lindau due to storage duration. *Bragantia*, *74*(4), 445-452.

Salvamani, S., Gunasekaran, B., Shaharuddin, N. A., Ahmad, S. A., & Shukor, M. Y. (2014). Antiartherosclerotic effects of plant flavonoids. *BioMed Research International*, *2014*.

Sargın, S. A., Akçicek, E., & Selvi, S. (2013). An ethnobotanical study of medicinal plants used by the local people of Alaşehir (Manisa) in Turkey. *Journal of ethnopharmacology*, *150*(3), 860-874.

Sari, A. O., & Tutar, M. (2010). Effects of Light, Cold Storage, and Temperature on Seed Germination of Golden Thistle (Scolymus hispanicus L.). *Journal of herbs, spices & medicinal plants*, *15*(4), 318-325.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. M., & Latha, L. Y. (2011). Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African Journal of Traditional, Complementary and Alternative Medicines*, *8*(1), 1-10.

Scalbert, A., & Williamson, G. (2000). Dietary intake and bioavailability of polyphenols. *The Journal of nutrition*, *130*(8), 2073S-2085S.

Shirai, A., Onitsuka, M., Maseda, H., & Omasa, T. (2015). Effect of polyphenols on reactive oxygen species production and cell growth of human dermal fibroblasts after irradiation with ultraviolet-A light. *Biocontrol science*, *20*(1), 27-33.

Soni, U., Brar, S., & Gauttam, V. K. (2015). Effect of seasonal variation on secondary metabolites of medicinal plants. *International Journal of Pharmaceutical Sciences and Research*, *6*(9), 3654.

Strüh, C. M., Jäger, S., Kersten, A., Schempp, C. M., Scheffler, A., & Martin, S. F. (2013). Triterpenoids amplify anti-tumoral effects of mistletoe extracts on murine B16. f10 melanoma in vivo. *PloS one*, *8*(4), e62168.

Sultana, M., Verma, P. K., Raina, R., Prawez, S., & Dar, M. A. (2012). Quantitative analysis of total phenolic, flavonoids and tannin contents in acetone and n-hexane extracts of Ageratum conyzoides. *International Journal of ChemTech Research*, *3*, 996-999.

Surek, E., & Nilufer-Erdil, D. (2014). Changes in phenolics and antioxidant activity at each step of processing from pomegranate into nectar. *International journal of food sciences and nutrition*, *65*(2), 194-202.

Szakiel, A., Pączkowski, C., Pensec, F., & Bertsch, C. (2012). Fruit cuticular waxes as a source of biologically active triterpenoids. *Phytochemistry Reviews*, *11*(2-3), 263-284.

Taylor, J. L. S., Rabe, T., McGaw, L. J., Jäger, A. K., & Van Staden, J. (2001). Towards the scientific validation of traditional medicinal plants. *Plant growth regulation*, *34*(1), 23-37.

Tiwari, P., Kumar, B., Kaur, M., Kaur, G., & Kaur, H. (2011). Phytochemical screening and extraction: a review. *Internationale pharmaceutica sciencia*, *1*(1), 98-106.

Tunalier, Zeynep., Kirimer, Nefle., Baser, Kemal Hüsnü Can. (2006). Demise of a 60 year old Turkish herbal medicine: Lityazol Cemil. International Congress of Ethnobotany (ICEB 2005), 213-216.

Tzin, V., & Galili, G. (2010). The biosynthetic pathways for shikimate and aromatic amino acids in Arabidopsis thaliana. *The arabidopsis book*, e0132.

Ugulu, I., Aydin, H., Yorek, N., & Dogan, Y. (2008). The impact of endemism concept on environmental attitudes of secondary school students. *Natura Montenegrina*, *7*(3), 165-173.

Ugulu, I., Baslar, S., Yorek, N., & Dogan, Y. (2009). The investigation and quantitative ethnobotanical evaluation of medicinal plants used around Izmir province, Turkey. *Journal of Medicinal plants research*, *3*(5), 345-367.

Ugurlu, E., & Secmen, O. (2008). Medicinal plants popularly used in the villages of Yunt Mountain (Manisa-Turkey). *Fitoterapia*, *79*(2), 126-131.

Vázquez, F. M., Suarez, M. A., & Pérez, A. (1997). Medicinal plants used in the Barros Área, Badajoz province (Spain). *Journal of Ethnopharmacology*, *55*(2), 81-85.

Vázquez, F. M., Suarez, M. A., & Pérez, A. (1997). Medicinal plants used in the Barros Área, Badajoz province (Spain). *Journal of Ethnopharmacology*, *55*(2), 81-85.

Viuda-Martos, M., Ruiz-Navajas, Y., Fernández-López, J., Sendra, E., Sayas-Barberá, E., & Pérez-Álvarez, J. A. (2011). Antioxidant properties of pomegranate (Punica granatum L.) bagasses obtained as co-product in the juice extraction. *Food Research International*, *44*(5), 1217-1223.

Wang, G. W., Lv, C., Shen, Y. H., & Zhang, W. D. (2017). Triterpenoids from Abies faxoniana and their cytotoxic activities. *Natural product research*, *31*(11), 1263-1269.

Wei, L., Zhang, W., Yin, L., Yan, F., Xu, Y., & Chen, F. (2015). Extraction optimization of total triterpenoids from Jatropha curcas leaves using response surface methodology and evaluations of their antimicrobial and antioxidant capacities. *Electronic Journal of Biotechnology*, *18*(2), 88-95.

Yang, C. S., Landau, J. M., Huang, M. T., & Newmark, H. L. (2001). Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annual review of nutrition*, *21*(1), 381-406.

Yang, D., Liu, X., Liu, M., Chi, H., Liu, J., & Han, H. (2015). Protective effects of quercetin and taraxasterol against H2O2-induced human umbilical vein endothelial cell injury in vitro. *Experimental and therapeutic medicine*, *10*(4), 1253-1260.

Yasuda, S., Yogosawa, S., Izutani, Y., Nakamura, Y., Watanabe, H., & Sakai, T. (2010). Cucurbitacin B induces G2 arrest and apoptosis via a reactive oxygen species-dependent mechanism in human colon adenocarcinoma SW480 cells. *Molecular nutrition & food research*, *54*(4), 559-565.

Zucco, F., Batto, A., Bises, G., Chambaz, J., Chiusolo, A., Consalvo, R.., & Fabre, G. (2005). An inter-laboratory study to evaluate the effects of medium composition on the differentiation and barrier function of Caco-2 cell lines. *Atla-Nottingham-*, *33*(6), 603.

Electronic References

http://www.apelasyon.com/Yazi/470-lityazol-cemil-ve-sevketi-bostan

National Institute of Health (URL: www.nih.gov)

APPENDIX A

CHEMICAL LIST

Chemical	Company
Ethanol	Tekkim
Folin	Sigma Aldrich
Sodium Carbonate	Sigma Aldrich
Sodium Nitrite	Sigma Aldrich
Aluminum Chloride	Sigma Aldrich
Sodium Hydroxide	Sigma Aldrich
Vanillin	Sigma Aldrich
Sulphuric Acid	Sigma Aldrich
ABTS	Sigma Aldrich
Potassium persulfate	Sigma Aldrich
Potassium phasphate monobasic	Sigma Aldrich
Potassium phasphate dibasic	Sigma Aldrich
Potassium Hydroxide	Riedel
Potassium phosphate dibasic	Sigma Aldrich
Nitric Acid	Sigma Aldrich
Potassium Iodide	Sigma Aldrich
Iodine	Sigma Aldrich
Pbs	Sigma-Aldrich
MEM Media	Sigma-Aldrich
Trypsin	Sigma-Aldrich
Calcium carbonate	Sigma-Aldrich
Ethyl acetate	Sigma-Aldrich
Chloroform	Sigma-Aldrich
Hexane	Sigma-Aldrich

APPENDIX B

STANDARD CALIBRATION GRAPHS

STANDARD CALIBRATION GRAPH FOR GALLIC ACID

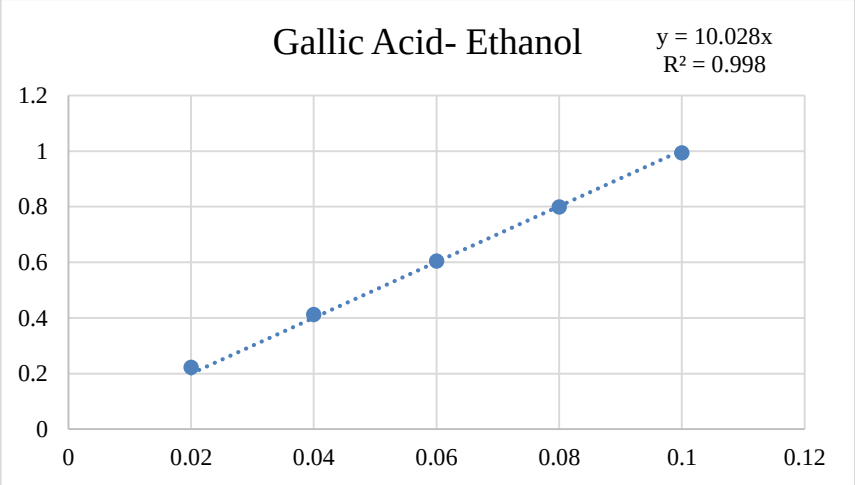


Figure B.1. Gallic Acid standard calibration curve by spectrophotometer

STANDARD CALIBRATION GRAPH FOR QUERCETIN

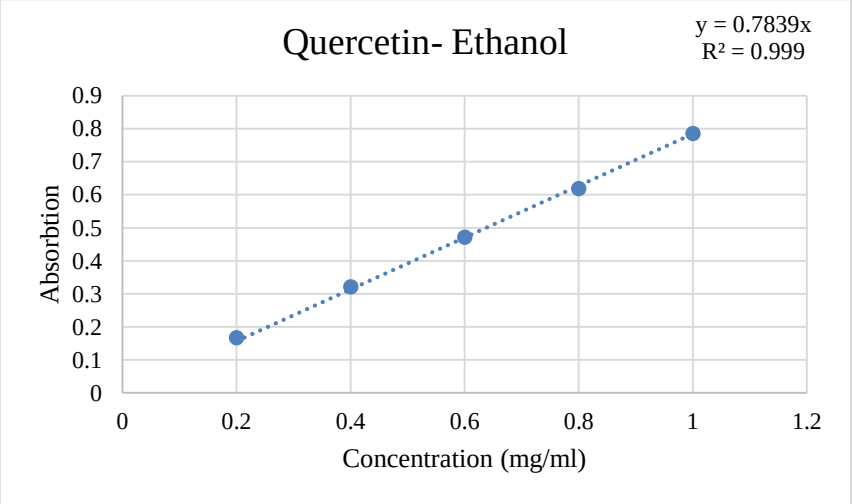


Figure B.2. Quercetin standard calibration curve by spectrophotometer

STANDARD CALIBRATION GRAPH FOR CATECHIN

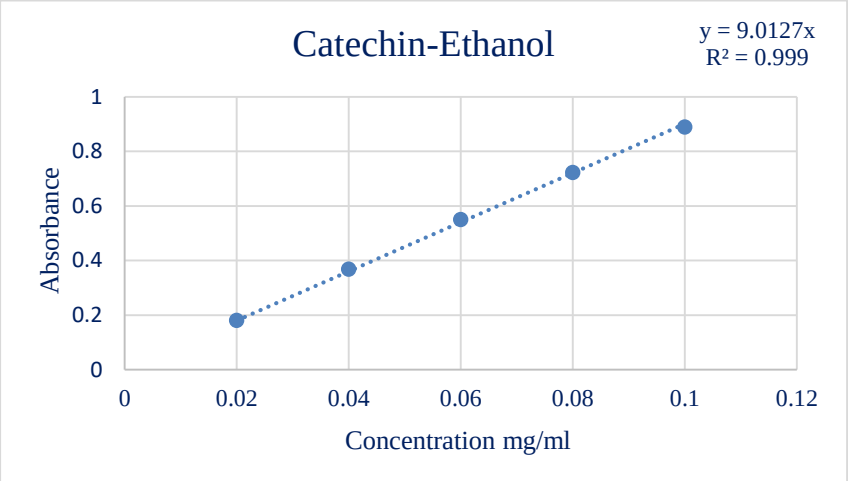


Figure B.3. Catechin standard curve by spectrophotometer

STANDARD CALIBRATION GRAPH FOR URSOLIC ACID

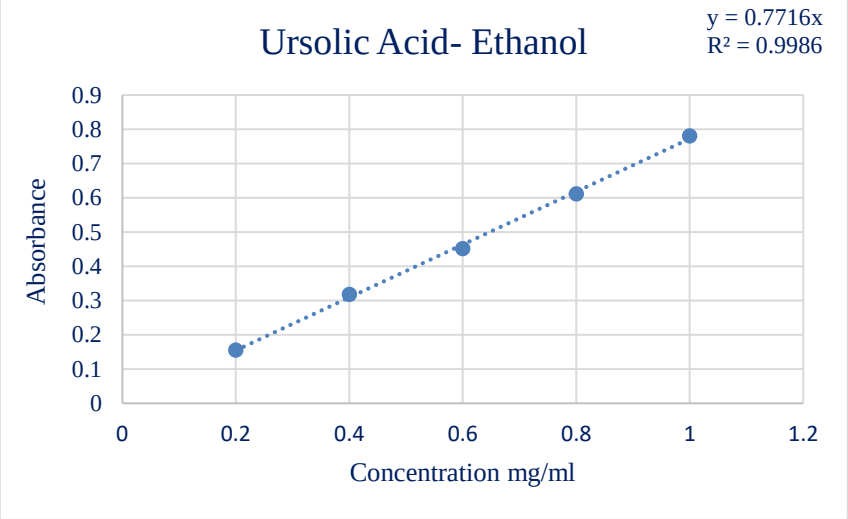


Figure B.4**.** Ursolic Acid standard calibration curve by spectrophotometer

STANDARD CALIBRATION GRAPH FOR TROLOX

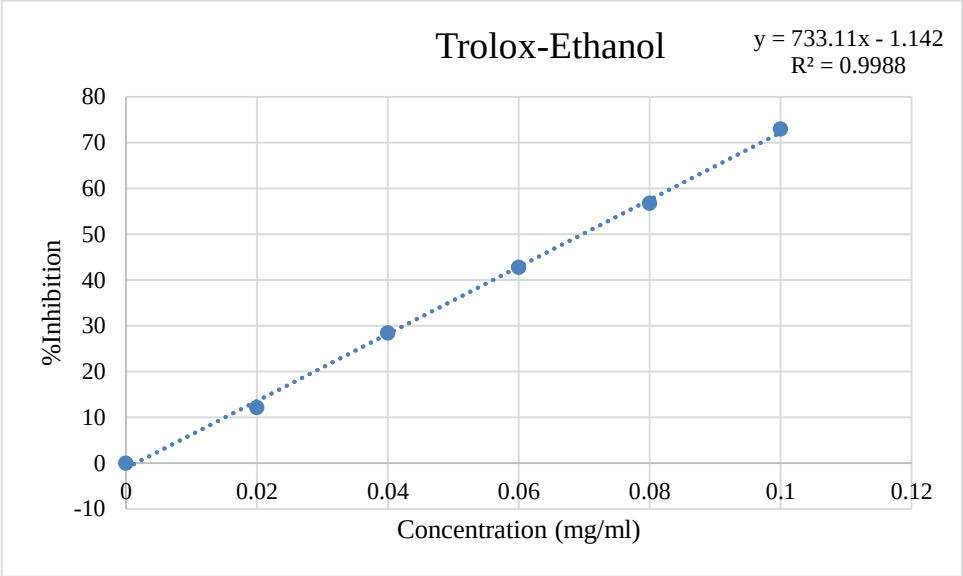


Figure B.5. Trolox standard calibration curve by spectrophotometer